Immune Modulation Mechanisms of Porcine Circovirus Type 2

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Abstract

Porcine circovirus associated disease (PCVAD) is an umbrella term for a multitude of diseases and syndromes that have a negative impact on the health and economics of pig production operations throughout the world. Porcine circovirus type 2 is the causative agent of PCVAD; however the presence of PCV2 alone is rarely enough to cause clinical disease. In order for the full development of PCVAD the presence of a co-infecting pathogen is required. The mechanisms by which co-infection leads to disease remain ongoing areas of research, but it is thought that host immune modulations by PCV2 or a co-infecting pathogen are critical in the pathogenesis of PCVAD. In the first study of this dissertation the ability of PCV2 to induce regulatory T-cells (Tregs) and alter cytokine production was evaluated *in vivo.* The addition of PCV2 to a multiple viral challenge resulted in a significant increase in Tregs. Levels of IL-10 and IFN-γ were also found to be altered when PCV2 was added to a multiple viral challenge. In further experiments, monocyte derived dendritic cells (MoDC) were infected with different combinations and strains of PCV2 and PRRSV *in vitro* and evaluated for expression levels of programmed death ligand-1 (PD-L1), IL-10, CD86, swine leukocyte antigen-1 (SLA-1), and swine leukocyte antigen-2 (SLA-2). Expression levels of PD-L1 were significantly increased in PCV2 and PRRSV co-infected MoDCs. SLA-1, SLA-2, and CD86 expression levels were significantly decreased in the MoDC treatment groups containing both PCV2 and virulent stains of PRRSV. MoDC IL-10 expression was significantly increased by PCV2 and virulent strains of

PRRSV co-infection. Finally, we investigated the role of the PD-L1/programmed death ligand-1 (PD-1) axis in porcine lymphocyte anergy, apoptosis, and the induction of Tregs. Lymphocyte populations with normal PD-1 expression had significantly higher percentages of anergic and apoptotic lymphocytes, and $CD4^{+}CD25^{High}FoxP3^{+}$ Tregs when compared to a PD-1 deficient lymphocyte population. The findings from these studies indicate host immune modulation by PCV2 *in vivo* and the development of a regulatory phenotype of dendritic cell following PCV2/PRRSV co-infections *in vitro* that may contribute to a dysfunctional adaptive immune response and the overall pathogenesis of PCVAD.

Dedication

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Attributions

Chapter 3: Addition of porcine circovirus type 2 (PCV2) to a multiple viral challenge induces regulatory T cells in pigs

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Chapter 4: PD-L1 expression is increased in monocyte derived dendritic cells in response to porcine circovirus type2 and porcine reproductive and respiratory syndrome virus infections

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Chapter 5: The PD-L1/CD86 ratio is increased in dendritic cells co-infected with porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus, and the PD-L1/PD-1 axis is associated with anergy, apoptosis, and the induction of regulatory T-cells in porcine lymphocytes

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Chapter 1: Introduction

Since its initial description in 1998 (Allan et al., 1998; Ellis et al., 1998) porcine circovirus type 2 (PCV2) has been the target of a great number of research endeavors. PCV2 is the causative agent of a group of diseases and syndromes occurring in pigs known collectively as porcine circovirus-associated disease (PCVAD). Although PCV2 is essential for the development of PCVAD, the most typical outcome of singular PCV2 exposure is a subclinical infection (Opriessnig and Halbur, 2012). For the full development of PCVAD to occur there is a need for a co-infecting pathogen. Several important viral and bacterial co-infecting pathogens have been identified thus far, perhaps none more prevalent than porcine reproductive and respiratory syndrome virus (PRRSV). PRRSV has been identified in several studies investigating PCVAD (Dorr et al., 2007; Drolet et al., 2003; Grau-Roma and Segales, 2007; Morandi et al., 2010; Wellenberg et al., 2004). In one study the presence of PRRSV was found in as many as 83% of the affected pigs (Wellenberg et al., 2004). A multitude of studies have investigated the interplay between PCV2 and PRRSV that leads to PCVAD, however the entire picture of pathogenesis remains elusive. The first aim of this dissertation examined the effect of the addition of PCV2 to a multiple viral challenge containing PRRSV on host cytokine responses and the induction of regulatory T-cells (Tregs) in an attempt to identify immune modulations that may potentiate disease *in vivo.* The addition of PCV2 resulted in a significant increase ($p<0.05$) in $CD4^{+}CD8^{+}CD25^{High}FoxP3^{+}$ and $CD4^{+}CD8^{-}CD25^{High}FoxP3^{+}$ Tregs 14 days post viral challenge (DPC). Levels of IL-10 and IFN-γ were also found to be altered when PCV2 was included in the viral challenge. Circulating IFN-γ levels were found to be significantly higher ($p<0.05$) in pigs with PCV2 in the viral challenge beginning on DPC 14 and continuing at DPC 21. IL-10 expression levels in the lungs of pigs containing PCV2 in the viral challenge were

found to be significantly higher ($p<0.05$) when compared to uninfected controls. The results of this first study implicate PCV2 in the initial immune suppression and subsequent immune stimulation that we hypothesize may be important in the pathogenesis of PCVAD.

One PCVAD of particular interest is PCV2 systemic disease (PCV2-SD). Formerly known as postweaning multisystemic wasting syndrome, PCV2-SD is a multifactorial disease with morbidity and mortality rates commonly found to range from 4-30% and 4-20% respectively, though both rates can occasionally be higher (Segales and Domingo, 2002). PCV2-SD is clinically noted by wasting ,weight loss, failure to gain weight, respiratory and gastrointestinal distress, and occasional jaundice (Segales, 2012). Because of the negative health and economic impact of PCV2-SD, a better understanding of the development and progression of the disease is needed. One of the hallmark lesions of PCV2-SD is histiocytic replacement of lymphoid tissue. During histiocytic replacement there is an infiltration of histiocytes into the lymph nodes, tonsils, and spleens of afflicted animals and a subsequent loss of lymphocytes (Allan et al., 1999; Ellis et al., 1999; Fenaux et al., 2004). Several mechanisms have been implicated in the depletion of lymphocytes in cases of PCV2-SD, including increased FasL activation, viral-induced lysis of lymphocytes, and the destruction of the lymphoid architecture (Darwich and Mateu, 2012). However, none of the proposed mechanisms have to date been experimentally confirmed as the responsible mechanism(s). In order to better understand the lymphocytic loss observed in cases of PCV2-SD, and attempt to address the co-infection necessity for the development of PCVAD, the second and third aims of this dissertation were directed towards investigation into the modulation of dendritic cells by PCV2 and PRRSV co-infections.

Dendritic cells (DCs) are important sentinels of the immune system that process and subsequently present antigens from foreign pathogens. Correct antigen presentation and lymphocyte activation by DCs is critical for an effective adaptive immune response. DC cytokine production and expression levels of surface molecules help in determining the type of immune response and the efficiency with which it will proceed (Banchereau and Steinman, 1998). Co-stimulatory surface molecules such as CD86, MHCI, and MHC II engage with their ligands on lymphocytes and trigger an active immune response. However, DC surface expression of regulatory surface molecules such as programmed death ligand-1 (PD-L1) can down regulate lymphocyte activation and lead to a suppression of the lymphocyte response (Selenko-Gebauer et al., 2003). Because of the importance of proper DC function in adaptive immune response development, and because both PCV2 and PRRSV are associated with DCs during the course of an infection (Darwich et al., 2010; Darwich and Mateu, 2012), the phenotype of DC that develops following co-infection is an important research topic. In the second aim of this dissertation monocyte derived dendritic cells (MoDC) were genotyped and phenotyped following PCV2 and/or PRRSV infections to determine expression levels of the regulatory marker PD-L1, and the stimulatory markers swine lymphocyte antigen-1 (SLA-1) and swine lymphocyte antigen-1 (SLA-2). The results showed that the expression levels of PD-L1 were significantly increased ($p<0.05$) in PCV2-infected MoDCs, as well as in PCV2 and PRRSV co-infected MoDCs. The MoDCs infected with PRRSV only also showed a strain-dependent increase in PD-L1 expression. SLA-1 and 2 expression levels were significantly increased ($p<0.05$) by PCV2 infection, and altered in the PRRSV, and PCV2/PRRSV co-infected MoDCs in a strain-dependent manner.

Expanding on the results from the second aim, the third aim of this dissertation further examined the phenotype of DC that occurs following PCV2/PRRSV co-infection by evaluating PD-L1, CD86, and IL-10 expression. As in the second aim, PD-L1 expression was significantly increased ($p<0.05$) in PCV2 and PCV2/PRRSV co-infected MoDCs, while singular PRRSV infection of MoDCs significantly increased ($p<0.05$) PD-L1 in a strain-dependent manner. CD86 expression was significantly increased ($p<0.05$) during singular PCV2 infection of MoDCs and significantly decreased ($p<0.05$) in the MoDC treatment groups containing both PCV2 and virulent stains of PRRSV. MoDC IL-10 expression was significantly increased ($p<0.05$) by singular PCV2 infection and PCV2 plus virulent strains of PRRSV co-infection. Taken together, the results from aim 2 and the first segment of aim 3 demonstrate the induction of a regulatory phenotype of MoDC following PCV2/PRRSV co-infection noted by decreased co-stimulatory marker expression, increased co-regulatory marker expression, and the highest levels of IL-10 expression. However, in cases of singular PCV2 exposure the observed significant increases in MoDC co-stimulatory marker expression may be enough for the proper activation of lymphocytes despite increases in IL-10 and PD-L1 expression. This may in part account for the reported functional T-cell responses in singular PCV2 infected subclinical pigs (Steiner et al., 2009). We hypothesize that the regulatory phenotype of DC as described in the PCV2/PRRSV co-infected MoDCs from the current experiments may be a mechanism of immune suppression in cases PCVAD pathogenesis.

The heightened MoDC PD-L1 expression observed in the above experiments raised questions about the possible effects of increased PD-L1 expression on lymphocyte responses in swine. Previous research in both murine and human species has demonstrated a role for the PD-L1/programmed cell death-1 (PD-1) axis in lymphocyte anergy, apoptosis, and the induction and

maintenance of Tregs (Amarnath et al., 2011; Francisco et al., 2009; Muhlbauer et al., 2006; Pen et al., 2014). To investigate the possible effects of increased MoDC PD-L1 expression and the PD-L1/PD-1 axis in swine, the final experiment of this dissertation utilized siRNA and the process of RNA interference to compare the responses of normal PD-1 expressing lymphocyte populations with the responses of PD-1 deficient ($PD\text{-}1^{(-)}$) lymphocyte populations. The results showed lymphocyte populations with normal PD-1 expression had significantly higher ($p<0.05$) percentages of anergic and apoptotic lymphocytes, and $CD4^{+}CD25^{High}FoxP3^{+}$ regulatory T-cells when compared to a $PD\text{-}1^{(-)}$ lymphocyte population. The description of the involvement of the PD-L1/PD-1 axis in swine lymphocyte anergy, apoptosis, and the induction of Tregs gives credence to the hypothesis that increased DC PD-L1 expression in cases of PCV2/PRRSV co-infections is a potential mechanism of immune suppression and lymphocyte depletion reported in cases of PCVAD.

Chapter 2: Literature Review

Introduction

Porcine circovirus associated disease (PCVAD) is currently one of the most economically important diseases facing the global pig industry. PCVAD encompasses several syndromes and diseases including, porcine circovirus type-2(PCV2)-systemic disease, pneumonia, enteritis, and reproductive failure (Opriessnig and Langohr, 2012). Reported cases of PCVAD are present in herds in every major swine producing country. It has been previously reported that PCVAD on average costs producers 3-4 dollars per pig, and up to 20 dollars per pig during outbreaks in the United States (Gillespie et al., 2009). Widespread vaccination programs for porcine circovirus type 2 (PCV2) in recent years have been effective at reducing PCV2 associated clinical disease in pig productions and have helped in reducing the economic impact of PCV2 (Opriessnig et al., 2012). Although the current PCV2 vaccines have had a positive impact on clinical disease, our understanding of the basic mechanisms involved in the pathogenesis of PCVAD is still lacking, and the threat of emerging strains or mutants of PCV2 highlight the need for continued research to combat future PCVAD outbreaks.

PCV2 Taxonomy and Genomic organization

Porcine circovirus (PCV) can be divided into two main types, the non-pathogenic porcine circovirus type-1 (PCV1), and the pathogenic porcine circovirus type-2. Both types of PCV's are circular, non-enveloped, DNA viruses in the *Circoviridae* family, genus *Circovirus* (Todd, 2004). PCV1 was the first type discovered as a contaminant of the pig kidney cell culture line, PK-15, in 1974 (Tischer et al., 1974). PCV2 was first described over 30 years later in 1998 (Allan et al., 1998; Meehan et al., 1998), and can be divided into at least three genotypes PCV2a,

PCV2b, and PCV2c. Prior to 2005, PCV2a was the predominant strain in swine herds throughout North America. However, in 2005-2006 a global shift was observed wherein PCV2b became the main genotype circulating throughout the world's swine herds (Gagnon et al., 2010; Lager et al., 2007; Meng, 2012; Patterson and Opriessnig, 2010). Although the shift to PCV2b was first observed in 2005-2006, a 2007 study analyzed 218 full length PCV2 genomes and determined the global shift to PCV2b dominance occurred prior to 2003(Dupont et al., 2008; Meng, 2012). PCV2c has been reported only from a few non-diseased herds in Denmark (Segales et al., 2008). In May of 2012 PCVAD cases were reported from a production farm that routinely vaccinated against PCV2. A mutant strain of PCV2b not previously seen in North America was determined to be the cause of the outbreak. The new mutant strain was 99.9% identical to a PCV strain isolated in China in 2010. This new mutant strain is thought to be more virulent than classical PCV2a or PCV2b and current vaccines may not provide full protection against the new strain(Opriessnig et al., 2013).

The genomic organization of PCV1 and PCV2 is very similar and the replication factors are interchangeable between the two viruses (Cheung, 2012). PCV2 produces 11 viral RNA's that have thus far been detected in infected cells, though their function has not been elucidated (Gao et al., 2014). Sequence analysis reveals a possible 8 open reading frames (ORFs) on the PCV2 genome, with two well described, functional ORFs. ORF 1 is in a clockwise orientation and encodes two replication proteins, Rep and Rep'. Rep is encoded by the entire ORF1 while Rep' is identical to Rep in the N-terminus portion but is spliced at the 3' portion to a different ORF (Cheung, 2012). Both Rep and Rep' localize to the nucleus and bind to the PCV2 origin of replication but they do so with different specificity (Finsterbusch et al., 2005; Steinfeldt et al., 2001). ORF 2 encodes the capsid protein and is found on the complimentary strand. Also found

on the complimentary strand is a putative ORF 3 that has been reported to be involved in cellular apoptosis (Liu et al., 2006; Liu et al., 2005). However, other laboratories have been unable to verify the role of ORF 3(Juhan et al., 2010). ORF 4 completely overlaps with ORF 3 and has a possible role inhibiting ORF 3 induced cellular apoptosis (Gao et al., 2014)

The different types and strains of PCVs vary in their genomic size and nucleotide sequence. PCV1 has a genome of 1759 nucleotides, PCV2a has a genome of 1768 nucleotides, and PCV2b has a genome of 1767 nucleotides (Trible and Rowland, 2012). PCV1 and PCV2 share approximately 70% sequence homology, while PCV2a and PCV2b share approximately 95% sequence homology (Trible and Rowland, 2012). The main difference between PCV2a and PCV2b occurs in the *cap* gene where the two types of PCV2 share approximately 90% sequence homology (Trible and Rowland, 2012).

PCV2 Life Cycle and Transmission

PCV2 infections occur in swine throughout the prenatal process and at different ages postnatally with differing outcomes and affected tissues (Segales et al., 2005). PCV2 infection of embryos leads to embryonic death and reabsorption by the sow (Mateusen et al., 2007). Viral tropism and replication in fetuses at 40-70 days of gestation is mainly in cardiomyocytes, hepatocytes, and monocytic cells (Sanchez et al., 2003). High replication in cardiomyocytes leads to heart failure of the fetus and mummification (Sanchez et al., 2004). After 70 days of gestation the replication rate of PCV2 declines considerably. Fetuses older than 70 days of gestation begin to develop a humoral immune response and the rate of mitosis decreases leaving PCV2 without a high number of dividing cells and the corresponding cellular polymerases needed for its own replication (Gassmann et al., 1988). In postnatal pigs PCV2 no longer has

tropism for cardiomyocytes and instead begins to effect lymphoblasts and monocytic cells. Lymphoblasts are fully susceptible to PCV2 and lead to a productive infection, while monocytic cells mainly take up virus particles but rarely lead to a productive infection (Lefebvre et al., 2008; Nauwynck et al., 2012; Sanchez et al., 2004). Although lymphoblasts and monocytic cells are the main targets for PCV2, replication has also been reported in epithelial and endothelial cells, and fibrocytes (Steiner et al., 2008).

PCV2 mainly utilizes glycosaminoglycans such as heparan sulphate and chondroitin sulphate B as cellular receptors for virus entry (Misinzo et al., 2006). Different cell types have different mechanisms for viral entry of PCV2. In certain monocytic cells PCV2 enters inefficiently via endocytosis in a clathrin dependent manner (Misinzo et al., 2005). Although this mechanism has been shown for the 3D4/31 monocytic cell line, the exact mechanism ofPCV2 entry into other monocytic cells such as macrophages, dendritic cells, and other monocyte lines has not yet been characterized (Meerts et al., 2005a; Meerts et al., 2005b; Steiner et al., 2008; Vincent et al., 2003). Epithelial cell entry can occur in one of two ways. First, PCV2 may be internalized via a dynamin and cholesterol independent, but actin and small GTPase dependent pathway leading to a productive infection. Secondly, PCV2 may enter epithelial cells through a clathrin dependent pathway that does not lead to a productive infection (Nauwynck et al., 2012)

Once inside the cell, disassembly of PCV2 again depends on the affected cell type. It is hypothesized that cell specific proteases active at different pH levels in different cell types can be used to explain the differing disassembly mechanisms observed (Nauwynck et al., 2012). Once uncoated, the single stranded viral genome is transported to nucleus where it is converted to a double stranded intermediate that serves as the template for Viral DNA synthesis and transcription (Cheung, 2012). Transcription is carried out by cellular enzymes and occurs in a

bidirectional manner with various viral RNA's produced through alternative splicing (Cheung, 2003a, b). Initiation of viral DNA synthesis occurs via a rolling circle mechanism at the viral genome origin of replication with required participation of the Rep-complex and a *cis*-acting element (Cheung, 2007; Mankertz et al., 1997). The subsequent elongation process is carried out by cellular enzymes as the Rep-complex contains no polymerase activity (Cheung, 2012). Currently the termination of PCV2 replication has not been determined conclusively (Cheung, 2012).

PCV2 can be shed or transmitted through a variety of routes. Horizontal transmission occurs mainly through direct contact with infected feces, urine, or infected animals via the oronasal route (Gillespie et al., 2009; Patterson and Opriessnig, 2010). Airborne spread of PCV2 has also been demonstrated as an effective transmission route. Air from a holding room containing PCV2 positive pigs was continuously pumped into a separate holding room with PCV2 naive pigs that subsequently became infected with PCV2 (Kristensen et al., 2013). Other exposure routes of PCV2 have been studied but the clinical and epidemiological impact of these routes remains to be established experimentally. A 2009 study found that pigs became infected with PCV2 after consuming uncooked meat from an infected pig (Opriessnig et al., 2009b). However; this direct oral route of consumption showed a delay in seroconversion suggesting that this route of infection may not be efficient (Rose et al., 2012). More experimental evidence is required to determine if transmission routes such as oral consumption, vaccine contamination, and fomite contamination pose a significant risk to pig herds (Rose et al., 2012).

Vertical transmission has also been established as an efficient transmission route of PCV2. Transplancental infection of fetuses has been demonstrated and occurs prior to, and after the development of fetal immune competence (Shen et al., 2010a). Vertical transmission through

infected semen has been shown to produce persistently infected or viremic piglets, and cause reproductive failure (Madson et al., 2009; Rose et al., 2007). Although these experiments demonstrated infected semen vertical transmission of PCV2, it is unclear if the amount of PCV2 shed under field conditions in boar semen would be sufficient to infect the fetus (Ladekjaer-Mikkelsen et al., 2001; Maldonado et al., 2005)

PCV2 associated diseases

The first case of PCV2 associated disease was described in Canada in 1991. The new disease was referred to as postweaning multisystemic wasting syndrome (PMWS), now referred to as PCV2 systemic disease (PCV2-SD), and first characterized by wasting and jaundice (Clark 1996; Harding 1996). PCV2-SD has since been further described to include conditions such as respiratory distress, diarrhea, enlarged subcutaneous lymph nodes, lymphocyte depletion with histiocytic replacement, and occasionally icterus (Krakowka et al., 2004; Rosell et al., 1999; Segales, 2012). Subsequent experiments and studies performed since the first reported case of PCV2-SD have implicated PCV2 in a variety of other conditions and diseases. PCV2 reproductive disease (PCV2-RD) is noted by late term abortions, still births, and mummification of the fetus (Brunborg et al., 2007; Madson et al., 2009; West et al., 1999). Although PCV2-RD is reproducible under experimental conditions, questions still remain about the frequency of PCV2-RD under field conditions (Segales, 2012). Porcine dermatitis and nephropathy syndrome (PDNS) affects pigs of all ages with mortality rates as high as 100% in pigs older than three months and a mortality rate of 50% in younger pigs (Segales, 2012). Although the mortality rate is high among affected pigs, PDNS is a rare disease with prevalence rates usually below 1% (Segales, 2012). Pigs suffering from PDNS can be anorexic, depressed, prostrate, and be

reluctant to move or have trouble doing so (Drolet et. al. 1999). The hallmark clinical sign of PDNS is the presence of irregular shaped, red or purple macules and papules on the skin, primarily on the hind limbs (Segales, 2012). It is important to note that some researchers have questioned the role and importance of PCV2 in the development of PDNS (Opriessnig and Halbur, 2012). PCV2 lung disease (PCV2-LD) is characterized by respiratory distress (Kim et al., 2003), and like PCV2 enteric disease (PCV2-ED) which is characterized by diarrhea (Kim et al., 2004; Opriessnig et al., 2007), must be diagnosed by histopathological findings because of the overlap with PCV2-SD. Examination of lung and gut tissues respectively is not enough to confirm PCV2-LD or PCV2-ED. In order to diagnosis PCV2-LD or PCV2-ED affected pigs must not have the microscopic lesions in lymphoid tissues that would be found in cases of PCV2-SD (Segales, 2012). Taken together, the above conditions and diseases have become collectively known as porcine circovirus disease (PCVD) in Europe, and porcine circovirus associated disease (PCVAD) in North America (Segales, 2012).

A new PCV2 related disease was recently described in pigs from farms that routinely vaccinate for PCV2. The newly described disease known as acute pulmonary edema (APE), is characterized by the rapid onset of respiratory distress followed almost immediately by death (Cino-Ozuna et al., 2011). Previously described PCVAD's are noted as slow, progressive syndromes. The peracute nature of APE differentiates the new disease from the previous PCVADs. In the 2011 study Cino-Ozuna et al. also did not identify any co-infecting pathogens playing a role in the pathogenesis of APE which is normally found in previously characterized PCVADs.

PCV2 infections are ubiquitous all around the world and the threat of PCVAD development is still a major concern to the pig industry, yet the most common result of PCV2

exposure is a subclinical infection (Segales, 2012). Retrospective studies have found the first known evidence of a PCV2 infection dates to Germany in 1962 (Jacobsen et al., 2009), while the first known diagnosis of PCVAD dates to the mid 80's (Jacobsen et al., 2009; Rodriguez-Arrioja et al., 2003). Although a sub-clinical infection may not lead to the development of PCVAD, sub-clinically infected pigs have been shown to have decreased porcine reproductive and respiratory syndrome virus (PRRSV) vaccine efficacy which can threaten the herd with PRRSV-induced pneumonia (Opriessnig et al., 2006).

Co-infecting pathogens in PCV2 associated diseases

PCV2 has been established as the essential agent in the development and pathogenesis of PCVAD (Bolin et al., 2001; Ellis et al., 1999; Ladekjaer-Mikkelsen et al., 2002). However, PCV2 alone is rarely enough to cause the development of PCVAD. Other co-infecting pathogens play a crucial role in the pathogenesis of PCVAD. Both viral and bacterial swine pathogens have been experimentally shown to contribute to PCVAD, and experimentally PCVAD is most efficiently replicated when PCV2 is joined by other swine pathogens in the challenge (Opriessnig and Halbur, 2012). Under natural conditions the presence of co-infecting pathogens in PCVAD cases is generally accepted as necessary for full disease development. A retrospective study found that of 484 systemic PCVAD cases in the Midwestern United State in 2000-2001 singular PCV2 infection was detected in only 1.9% of the studied cases (Pallares et al., 2002). To date no single co-pathogen has been identified as the solely responsible for increasing the severity of PCVAD (Opriessnig and Halbur, 2012), and in fact many pathogens have demonstrated the ability to increase the severity of PCVAD.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive sense, single stranded RNA virus from the family *Arterivirdae*, genus *Arterivirus*, order *Nidovirales* (Cavanagh, 1997; Meulenberg et al., 1993). PRRSV is arguably the most prominent co-pathogen in cases of PCVAD. In a 2004 study PRRSV was identified in 83% of the PCVAD cases examined (Wellenberg et al., 2004). Several experiments have evaluated the relationship between PRRSV and PCV2 in cases of PCVAD. Combined PCV2-PRRSV infections have been shown to greatly increase the severity of clinical disease in PCVAD cases (Harms et. al. 2002), and PRRSV has been shown to prolong the shedding and enhance the replication of PCV2 (Allan et al., 2000; Rovira et al., 2002; Sinha et al., 2010).Other viral pathogens such as torque teno sus, swine influenza, pseudorabies, and porcine epidemic diarrhea virus have all been implicated as possible co-pathogens in cases of PCVAD (Dorr et al., 2007; Jung et al., 2006; Kekarainen et al., 2006; Opriessnig and Halbur, 2012; Pallares et al., 2002). Several types of bacteria have also been identified as co-pathogens leading to an increase in the severity of PCVAD. *Mycoplasma hyopneumoniae* co-infections are perhaps the most well studied bacterial co-pathogen in cases in PCVAD. In a 2011 field study of 147 pig farms seropositivity to *Mycoplasma hyopneumoniae* was identified as a factor correlating to increased PCVAD severity (Alarcon et al., 2011) Other experiments and studies involving PCV2-*Mycoplasma hyopneumoniae* co-infections have found that the presence of *Mycoplasma hyopneumoniae* in the pathogen challenge leads to increased PCV2 replication and longer persistence of PCV2 antigen (Opriessnig and Halbur, 2012; Opriessnig et al., 2011; Opriessnig et al., 2004).

The exact mechanism by which a co-pathogen enhances the severity of PCVAD is yet to be elucidated, but several theories have been proposed. PCV2 replication is completely dependent on new cellular DNA synthesis in the infected cell (Kennedy et al., 2000), therefore

co-pathogen induced cellular proliferation is one mechanism that may give PCV2 the cellular environment needed for efficient and increased replication (Opriessnig and Halbur, 2012). Cytokine response alteration is another proposed mechanism of enhanced PCV2 replication. For example, a recent study involving PCV2 and *Mycoplasma hyopneumoniae* co-infections found that *Mycoplasma hyopneumoniae* increased IFN-γ and IL-10 production, while lowering the IFN-α response (Zhang et al., 2011). This cytokine environment correlated with increased severity of PCVAD (Opriessnig and Halbur, 2012). Immune suppression may also play a role in the persistence of PCV2 infections and the severity of PCVAD. PRRSV is one co-pathogen that is suspected of inducing immune suppression in cases of PCVAD. Precisely how PRRSV-induced immune modulations and suppression increase PCV2 replication and persistence is not known, but PRRSV-induced increases in regulatory T-cells populations (Silva-Campa et al., 2012), and PRRSV-induced expression of Fas and Fas ligand on lymphocytes and macrophages (Chang et al., 2007) are two proposed mechanisms. PRRSV has previously been shown to increase the pathogenicity of other respiratory viruses through immune suppression and alteration mechanisms (Jung et al., 2009).

Porcine Immune Response to PCV2

The type, strength, and efficiency of an immune response are critical in protecting the host against any pathogen. In the case of PCV2 infections, this response is shaped in part by the fact that PCV2 directly infects cells of the immune system or cells closely associated with lymphoid tissue such as stromal cells(Darwich and Mateu, 2012; Steiner et al., 2008). This direct interaction with the immune system is mainly associated with PCV2 presence in antigen presenting cells (APC's) such as dendritic cells, monocytes, and macrophages of the innate

immune system. PCV2 antigen and DNA have been identified in APC's, however APC's are reportedly not efficient hosts for PCV2 replication (Chang et al., 2006; Gilpin et al., 2003; Perez-Martin et al., 2007; Steiner et al., 2008; Vincent et al., 2005; Vincent et al., 2003). Experiments involving PCV2-macrophage interactions have reported that macrophages readily internalize PCV2 with no effect on the phagocytic ability of the cells and a lack of detectable viral replication (Chang et al., 2006). Even without productive replication, PCV2 did have an effect on macrophage cytokine production. The presence of PCV2 increases macrophage production of tumor necrosis factor-α and interleukin-8, and the mRNA levels of macrophage-derived chemotactic factor-II, granulocyte colony-stimulating factor, and monocyte chemotactic protein-1 (Chang et al., 2006). The presence of PCV2 in macrophages also inhibits the microbicidal activity of the cells. Macrophages infected with PCV2 demonstrated a reduced capacity to destroy *Candida albicans*, most likely through impaired production of O_2- and H_2O_2 (Chang et al., 2006).

In dendritic cells (DC), as in macrophages, there is a reported lack of productive PCV2 replication (Vincent et al., 2003). Even with a lack of productive replication PCV2 can have important and differing modulatory effects on DC's. PCV2 immune modulations in DC's are largely dependent on the DC subset. In myeloid DC's (mDC) PCV2 has the ability to persist without causing apoptosis or altering the surface protein phenotype of the cells (Darwich and Mateu, 2012). Less efficient pathogen internalization has been reported in mDC's with PCV2 present (Balmelli et al., 2011), however mDC maturation and cytokine production appear to be unaffected by PCV2 (Vincent et al., 2005). The harboring of PCV2 in mDC's has been suggested as a mechanism of PCV2 spread and transmission (Vincent et al., 2003). In bone marrow derived dendritic cells (BMDC), PCV2 has been shown to alter the cytokine response dramatically.

PCV2 shut down the interferon alpha response in BMDCs co-infected with pseudorabies virus and also strongly induced IL-12 (Kekarainen et al., 2008a).Plasmacytoid dendritic cells (pDC's) are another subset of DC's that produce particularly high amounts of type 1 interferons in response to viral or bacterial pathogens via pattern recognition receptors. pDC's are important in shaping the initial innate immune response as well as the adaptive immune response (Vincent et al., 2007). The presence of PCV2 in pDC's inhibits their typically high production of type 1 interferons, as well as the production of other important cytokines such as tumor necrosis factor-alpha (Vincent et al., 2005). This type of inhibited cytokine production is thought to impair mDC maturation in response to pathogen challenge and subsequently alter the adaptive response as well (Takeda et al., 2003).

Several studies have found that the viral DNA is responsible for a majority, though not all, of the observed PCV2 immune modulations (Hasslung et al., 2005; Kekarainen et al., 2008a; Kekarainen et al., 2008b; Vincent et al., 2007; Wikstrom et al., 2011; Wikstrom et al., 2007). PCV2 CpG-ODN's have been shown to alter cytokine production in APCs and peripheral blood mononuclear cell (PBMC) populations (Kekarainen et al., 2010). For example, PCV2 CpG-ODN's are responsible for the silencing of the type 1 interferon response in stimulated pDC's discussed previously (Vincent et al., 2007). The double stranded (ds) intermediate replicative form of the PCV2 genome has also been demonstrated as immunomodulatory. PCV2 dsDNA was found to interfere with cytoskeletal rearrangements in pDC's, and reduce the endocytosis efficiency of myeloid DC's (Balmelli et al., 2011). Although a majority of PCV2 induced immune modulations discovered thus far been have been attributed to PCV2 DNA, viable PCV2 virions also have the ability to alter immune function. Viable PCV2 virions were shown to

induce IL-10 production in PBMCs, while virus like particles and PCV2 CpG-ODN's failed to do so (Kekarainen et al., 2008a).

Immune modulations that occur during co-infection are of particular interest for their contribution to the pathogenesis of PCVAD. PCV2-SD afflicted pigs often have altered cytokine responses as one mechanism of immune modulation. PBMC from PCV2 infected pigs have a decreased capacity to produce IL-2, IL-4, and IFN-γ, thus hampering the overall immune response (Darwich et al., 2003a). Increased IL-10 production in lymphoid tissue and PBMC populations is a common finding in pigs suffering from PCV2-SD (Darwich et al., 2003a; Doster et al., 2010; Kim and Chae, 2004; Sipos et al., 2004). Pigs suffering from PVC2-SD have demonstrated elevated levels of IL-10 in their thymus (Darwich et al., 2003b), mandibular lymph node, spleen, and tonsils (Doster et al., 2010) and their serum (Hasslung et al., 2005; Stevenson et al., 2006). IL-10 is produced by a range of cell types and is an immunosuppressive cytokine with a high degree of anti-inflammatory activity (Doster et al., 2010). High levels of IL-10 production in virally infected hosts have been associated with inhibited viral clearance leading to viral persistence (Quintana et al., 2001). Based on experimental evidence it appears that IL-10 and its immunosuppressive activity may have an important role in the pathogenesis of PCVAD (Darwich and Mateu, 2012).

The development of PCVAD is the worst case scenario in PCV2 exposure, but it is not the most common. As mentioned before, a vast majority of PCV2 exposures lead to subclinical infections. In cases of PCV2 sub-clinical infections host protection is mediated by a combination of neutralizing antibody (NA) production and cell mediated immune responses (Darwich and Mateu, 2012). NA levels in PCV2 infected pigs have been directly correlated with viral replication (Meerts et al., 2005c) and clinical outcome (Fort et al., 2007). The Cap protein of

PCV2 is the most immunogenic and contains neutralizing epitopes that provide NA protection (Blanchard et al., 2003; Darwich and Mateu, 2012). Cell mediated response to PCV2 is has been determined experimentally through monitoring the levels IFN-γ secreting cells. The development of both $CD4^+$, and $CD8^+$, IFN-γ secreting T-cells occurs during PCV2 infection and most likely contributes to host protection (Fort et al., 2009b; Steiner et al., 2009). Both the Cap and Rep proteins of PCV2 contribute to cell mediated protection in subclinical cases of PCV2 infection (Fort et al., 2010). The duration of host protection provided after natural infection or vaccination has not been elucidated conclusively (Darwich and Mateu, 2012). One experiment found that the presence of NA was still detectable three months after vaccination and still provided protection against the development viremia (Opriessnig et al., 2009a).

Regulatory T-cells

Regulatory T-cells (Treg) are critical cells of the immune system that help to prevent chronic inflammation, prevent autoimmune disease, and promote peripheral tolerance to non-harmful and self-antigens. The immuno suppressive functions of Tregs are critical to maintaining cellular homeostasis but they can also have negative effects during viral infection. Numerous studies have found that an increase in Tregs can limit viral clearance and be detrimental to the clinical outcome (Andersson et al., 2005; Belkaid, 2007; Boettler et al., 2005; Rouse et al., 2006; Rushbrook et al., 2005; Suvas et al., 2003). The importance of Tregs has made them the focus of many research endeavors over the past 15 years, though the full scope and function of Tregs in immune system modulations has yet to be fully characterized.

Tregs can be generally separated into two types, natural Tregs (nTregs), and inducible Tregs (iTregs) (Kaser et al., 2012). nTregs develop in the thymus, while iTregs are a less well characterized subset of Tregs that develop in the periphery in response to certain cytokine environments or prolonged antigen exposure. nTregs are phenotypically defined by expression of CD4, high levels of CD25, the transcription factor forkhead box P3 (FoxP3), and have a T-cell receptor repertoire biased towards self-antigens (Fontenot et al., 2003; Francisco et al., 2009; Hori et al., 2003; Vignali et al., 2008). iTregs also express FoxP3 but lack a further common phenotype; they develop in both the CD4+ and CD8+ T-cell subsets, and are characterized by their suppressive functions and the production of suppressive cytokines such as IL-10 (Kaser et al., 2012; Mills, 2004). It is important to note that while expression of FoxP3 is essential for Treg development, in human cells FoxP3 expression is not limited to Tregs, therefore FoxP3 expression alone is not sufficient to characterize Tregs. A significant portion of human T-cells can express FoxP3 while possessing no suppressive activity (Allan et al., 2007; Gavin et al., 2006; Morgan et al., 2005; Tran et al., 2007; Vignali et al., 2008; Wang et al., 2007). Although both Treg subsets have immune suppression functions, it has been proposed that the responsibilities of the two subsets are independent of one another. nTregs are proposed to have more of a role in preventing autoimmune diseases, while iTregs are involved in limiting chronic inflammation and play a major role during infections (Curotto de Lafaille and Lafaille, 2009). An interesting overlap between the two Treg subsets is the activation of iTregs by nTregs. Activated nTregs have been shown to produce immunosuppressive cytokines which alter the immune environment in the periphery and lead to the induction of iTregs (Andersson et al., 2008).

There is a wide variety of proposed mechanisms of immune suppression carried out by Tregs. Below is a summary of Treg mediated suppressive mechanisms as reviewed by Vignali et.

al. 2008 (Vignali et al., 2008). Treg mediated suppression through the production of IL-10 and TGF-β has been the focus of many research efforts in recent years. Although IL-10 and TGF-β are well established immunosuppressive cytokines, there is conflicting data regarding the necessity of these cytokines to Treg function. Recent *in vivo* studies have demonstrated the importance of IL-10 producing Tregs in the control of asthma and allergic reactions through immune response dampening and TGF-β induction (Hawrylowicz and O'Garra, 2005; Joetham et al., 2007). However, *in vitro* studies have found that IL-10 and TGF-β production by Tregs was not essential to immune suppression function. (Dieckmann et al., 2001; Takahashi et al., 1998; Thornton and Shevach, 1998). Regardless of the direct role of secreting immunosuppressive cytokines by Tregs, IL-10 and TGF-β are known to induce iTregs thereby contributing to Treg immune suppression (Andersson et al., 2008; Vignali et al., 2008).

Another proposed mechanism of Treg immune suppression is cytolysis of targeted cells. Treg mediated cytolysis has been shown to occur through the production of granzyme B and perforin (Grossman et al., 2004), through the TRAIL-DR5 pathway (Ren et al., 2007), and through galectin production in a granzyme and perforin independent manner (Toscano et al., 2007).

Metabolic disruption is a group of proposed suppression mechanisms that alter the survival, proliferation, and inhibitory signals received by target effector T-cell populations. Sequestration of IL-2 by Tregs expressing a high level of CD25 has long been a proposed mechanism for Treg inhibition. As with many topics involving Treg research, there is debate as to whether IL-2 consumption by Tregs is, by itself, sufficient for immune suppression. One study found that cytokine deprivation, particularly IL-2 deprivation, mediated effector T-cell apoptosis (Pandiyan et al., 2007). However this also included the depletion of other cytokines,

and Oberle et. al. have demonstrated that IL-2 deprivation is not required for Treg mediated immune suppression (Oberle et al., 2007). Another suppression mechanism that is included in the metabolic disruption group is the production of adenosine nucleosides by Tregs. Pericellular adenosine binding with the A2A receptor on effector T-cells was shown to suppress target cell function, and also increase Treg populations by altering the cytokine production of the effector T-cells from IL-6 production to TGF-β production (Deaglio et al., 2007; Zarek et al., 2008). A final mechanism of Treg suppression that falls under the metabolic disruption category is the transfer of cyclic AMP from Tregs to effector T-cells through membrane gap junctions. Tregs harbor a large amount of cyclic AMP, and upon transfer to neighboring effector T-cells the cyclic AMP inhibits proliferation and IL-2 production in the target cell (Bodor et al., 2012; Bopp et al., 2007).

The previously discussed Treg suppression mechanisms have focused on direct Treg suppression of effector T-cells. However, Treg manipulation of dendritic cell function is also a proposed mechanism of Treg immune suppression. Tregs constitutively express cytotoxic T-lymphocyte antigen 4 (CTLA4) which when engaged with CD80 or CD86 on DC's suppresses DC activation of effector T-cells by downregulation of stimulatory molecules on the DC's surface (Oderup et al., 2006; Read et al., 2000; Serra et al., 2003). CTLA4 engagement to CD80/86 on DC's also causes DC's to express indoleamine 2,3-dioxygenase (IDO) which modulates tryptophan metabolism by the DC's suppressing the downstream activation of effector T-cells (Fallarino et al., 2003; Mellor and Munn, 2004). Downregulation of stimulatory molecules such as CD80/CD86, or B7-H4 on DC's by Tregs has been shown in several experiments, though the exact mechanism of this DC phenotype alteration has yet to be elucidated (Cederbom et al., 2000; Kryczek et al., 2006; Vignali et al., 2008). Suppression of

DC maturation and antigen presenting capabilities by Tregs has also been observed experimentally. One mechanism of interest in blocking DC maturation is Treg expression of lymphocyte-activation gene 3 (Lag3). Lag3 binds to MHC II molecules on immature DC's and induces an inhibitory pathway in the DC's leading to stunted maturation and immunostimulatory capacity (Liang et al., 2008).

Porcine Tregs

Porcine Tregs, like human and murine Tregs, are noted by their expression of the transcription factor FoxP3 and occur in both iTreg and nTreg classes (Kaser et al., 2012). Porcine Tregs were first characterized both phenotypically and functionally in 2008 (Kaser et al., 2008). Since their first description several research endeavors have focused on uncovering the suppressive mechanisms employed by porcine Tregs. In general, porcine Tregs utilize the same main classes of suppression as human and murine Tregs; cell-cell contact dependent mechanisms, the production of soluble suppressive factors, and competition for growth factors (Kaser et al., 2011). However, research in to porcine Tregs is still in the early stages and exact mechanisms of suppression proven in other species have yet to be tested in porcine Tregs.

The role of Tregs during swine infections is an ongoing field of research. To date, the role of Tregs in bacterial and parasitic swine infections has yet to be fully elucidated. Viral infection impact of porcine Tregs has been most comprehensively studied in PRRSV infection models. PRRSV has been shown to induce Tregs during infection, thereby contributing to disease progression and the persistent nature of a PRRSV infection (LeRoith et al., 2011; Silva-Campa et al., 2010; Silva-Campa et al., 2009; Wongyanin et al., 2010). Co-infection of DC's with PCV2a and PRRSV in an *in vitro* model of PCVAD have also shown an increase in the

percentage of Tregs (Cecere et al., 2012). The role of Tregs during viral infections could be profound as immune system modulations, particularly immune suppression, are suspected mechanisms leading to decreased clinical outcomes during swine viral infection outbreaks.

Programmed Death Ligand-1

Programmed death ligand-1 (PD-L1), also known as cluster of differentiation 274, and B7-H1, is a type 1 transmembrane protein with immune suppression activities (Freeman et al., 2000; Jeon et al., 2007). PD-L1 and its ligand, programmed death-1 (PD-1), have emerged as important research and therapy targets because of their role in disease through immune suppression. PD-L1/PD-1 binding has been implicated in immune tolerance and suppression during tumor development, pregnancy, tissue grafts, autoimmune disease, and infections (Freeman et al., 2000; Koga et al., 2004; Pen et al., 2014; Polanczyk et al., 2006; Shi et al., 2011). PD-L1 engagement with PD-1 on effector T-cells blocks T-cell receptor signaling by inhibiting phosphatidylinositol 3-kinase activation and recruiting Src homology region 2 domain-containing phosphatase -1 (SHP-1) and SHP-2 leading to T-cell dysfunction and exhaustion (Chemnitz et al., 2004; Francisco et al., 2010). PD-L1 is expressed on DC's, macrophages, activated T and B-cells, and a variety of other tissues. The role of PD-L1 appears to be cell type dependent and all the activities of PD-L1 in different settings are not yet entirely clear, though PD-L1 expression on non-lymphoid tissues is hypothesized to play a role in determining the extent of immune responses at local sites of inflammation, and increased PD-L1 expression on antigen presenting cells has been experimentally established as an immune suppression mechanism (Freeman et al., 2000; Jeon et al., 2007; Pen et al., 2014)

PD-L1 and viral infections

Dysfunction of antigen-specific T-cells is one characteristic commonly found during viral infections that aides in virus survival and increases disease severity. One of the phenotypic hallmarks of impaired T-cell function during both chronic and acute viral infections is increased PD-1 expression (Barber et al., 2006; McNally et al., 2013). Increased levels of PD-1 expression on T-cells increases engagement with PD-L1 found on many cell types and tissues. During adenoviral and hepatitis B infections PD-L1 is up-regulated on hepatocytes leading to decreased T-cell function, T-cell apoptosis, and lowered viral clearance (Muhlbauer et al., 2006). A study by McNally et. al. in 2013 found that airway epithelial cells increase expression of PD-L1 upon infection with influenza A virus increasing the viral load and slowing disease recovery. Interference with the PD-L1/PD-1 axis by PD-L1 blocking antibody returned T-cell function and improved disease recovery (McNally et al., 2013). Numerous studies examining a multitude of viruses such as human immunodeficiency virus, chronic lymphocytic choriomeningitis virus, hepatitis B, and hepatitis C have also shown that PD-L1/PD-1 binding is detrimental to the host during infections and a subsequent blockade of PD-L1 leads to returned T-cell function and better viral clearance (Barber et al., 2006; Boni et al., 2007; Day et al., 2006; Urbani et al., 2006; West et al., 2013).

Viral infections can influence the expression of PD-L1 besides direct infection of the target cell through alterations in the production of Type 1 interferons. Experimental evidence has shown that PD-L1 expression levels increase in the presence of Type 1 interferons in both human and murine models (Muhlbauer et al., 2006; Teijaro et al., 2013; Wilson et al., 2013). Because of this, viruses that alter the production of type 1 interferons have direct impact on the PD-L1/PD-1 axis and the downstream immune system modulations. Plasmacytoid dendritic cells

(pDCs), also known as natural interferon producing cells, produce particularly high amounts of type 1 interferons upon stimulation by an invading pathogen. Type 1 interferon production by pDCs is crucial to shaping the host immune response. Several viruses have been experimentally shown to alter pDC type 1 interferon production, which not only affects overall NK, and T and B cell responses, but can also directly inhibit or induce PD-L1 expression (Colonna et al., 2004; Liu, 2005; Teijaro et al., 2013). PCV2 has been shown to shut down the type 1 interferon response in pDCs, while PRRSV has been shown to alter the pDC type 1 interferon response by either inducing or suppressing type 1 interferon production in a strain dependent manner (Baumann et al., 2013; Vincent et al., 2007; Vincent et al., 2005). These observed modulations to the type 1 interferon response in pDCs may directly contribute the pathogenesis of both viruses through altered PD-L1 expression levels.

PD-L1 and DC's

Proper dendritic cell (DC) function is critical to a host's ability to mount an effective immune response against an invading pathogen. During an infection DCs capture and process antigens in order to coordinate the adaptive immune response. DC cytokine production and expression levels of surface molecules help in determining the type of immune response and the efficiency with which it will proceed (Banchereau and Steinman, 1998). Co-stimulatory surface molecules such as CD80, CD86, MHC II, and CD40 engage with their ligands on lymphocytes and trigger an active immune response. However, DC surface expression of regulatory surface molecules such as PD-L1 can down regulate lymphocyte activation and lead to immune suppression. This type of immune suppression aides in preventing autoimmune diseases and excessive inflammation as an infection proceeds, but can also be utilized by viral pathogens as a mechanism to evade an effective lymphocyte response. The balance between expression levels

of stimulatory and regulatory surface molecules can determine a DCs immune function and phenotype as stimulatory or regulatory (Wolfle et al., 2011). Regulatory DCs mature in an immune environment that favors expression of regulatory surface molecules, such as prolonged exposure to IL-10, TGFβ, TNF-α, or certain toll like receptor agonists (Faunce et al., 2004; McGuirk et al., 2002; Menges et al., 2002; Wolfle et al., 2011). These regulatory DCs have been phenotypically characterized in human and murine models by their production of IL-10 and high expression levels of regulatory molecules such as PD-L1, PD-L2, ILT3, and ILT4 (Gordon et al., 2014). Differentiation into regulatory DCs occurs via STAT-3 signaling, which when experimentally blocked reduces regulatory surface molecule expression (Wolfle et al., 2011).

High DC expression levels of PD-L1 have been shown experimentally to have a negative impact on T-cell function (Selenko-Gebauer et al., 2003). An increase in overall PD-L1 expression along with an increased PD-L1/CD86 ratio impairs DC function during hepatitis C infections leading to reduced stimulatory capacity and T-cell exhaustion (Shen et al., 2010b). Another study found that blocking the activity of PD-L1 on DCs during exposure to influenza virus nuclear protein 1 returned function and cytokine production to exhausted antigen-specific T-cells (Pen et al., 2014).

PD-L1 and Tregs

Both regulatory T-cells and high levels of PD-L1 expression are immune suppression mechanisms that maintain peripheral tolerance but can also have a negative impact during the course of infections. Historically, the presence of cytokines such as IL-10 and TGF-β were the most well established mechanisms of Treg induction. Recently however the PD-L1/PD-1 axis has been experimentally established as a potent inducer of Tregs and maintainer of Treg function

in murine and human models (Amarnath et al., 2011; Francisco et al., 2009). In the murine model PD-L1 coated beads were able to induce iTregs *in vitro*, enhance and sustain FoxP3 expression, and prevent fatal inflammatory disease that was observed in a PD-L1 knockout model (Francisco et al., 2009). PD-L1/PD-1 binding has also been shown to convert Th1 cells into the Treg phenotype in an *in vivo* model of human-into-mouse xenogeneic graft-versus-host disease (Amarnath et al., 2011). Interestingly, besides inducing and maintaining Tregs, PD-L1 has also been shown to negatively regulate Treg function. By disrupting STAT-5 phosphorylation, PD-L1 engagement with Tregs expressing high levels of PD-1 isolated from the livers of patients chronically infected with hepatitis C led to lowered Treg proliferation, and a decrease in the expression of immune suppression surface markers (Franceschini et al., 2009).

Chapter 3: Addition of porcine circovirus type 2 (PCV2) to a multiple viral challenge induces regulatory T cells in pigs

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Abstract

Porcine circovirus type 2 (PCV2) is the primary and essential causative agent of porcine circovirus associated disease (PCVAD), however, the development of clinical PCVAD typically requires co-infection with other bacterial or viral swine pathogens. In this study we hypothesized that viral co-infection with PCV2 in pigs would result in an increase in T-reg percentages in peripheral blood mononuclear cells (PBMC), increased IL-10 and TGF-β, decreased INF-γ levels in serum and tissue, and decrease the ability of PBMC's to proliferate. Unvaccinated, PCV2a/2b+PRRSV+Porcine parvovirus type 1(PPV1) infected pigs had significantly ($p<0.05$) increased T-regs, decreased circulating TGF-β, and increased circulating IFN-γ. Analysis of cytokine mRNA expression revealed a significant increase ($p<0.05$) in the relative expression of

IL-10 levels in the lung. Unvaccinated, pigs infected with PPV1+PRRSV alone, had significantly ($p<0.05$) lower percentages of T-regs, significantly increased circulating IL-10 and IFN-γ, and significantly lower circulating TGF-β. Cytokines and T-reg percentages from pigs vaccinated with a modified live-attenuated PCV2 based vaccine were unchanged compared to the unvaccinated, uninfected, controls. The results of this study showed an induction of T-regs *in vivo* when PCV2 was added to the viral challenge in the unvaccinated but not in the vaccinated pigs. The observed increase in T-regs represents one possible mechanism in the pathogenesis of clinical PCVAD.

Introduction

Porcine circovirus associated disease (PCVAD) is currently one of the most economically important diseases facing the global pig industry. PCVAD encompasses several syndromes and diseases including, Porcine circovirus type-2(PCV2)-systemic disease, pneumonia, enteritis, and reproductive failure (Opriessnig and Langohr, 2012). Reported cases of PCVAD are on the rise, and are present in herds in every major swine producing country. It has been previously reported that PCVAD on average costs producers 3-4 dollars per pig, and up to 20 dollars per pig during outbreaks (Gillespie et al., 2009).

PCV2 is the primary and essential causative agent of PCVAD. PCV2 is a circular, non-enveloped, DNA virus in the *Circoviridae* family, genus *Circovirus*(Todd, 2004). PCV2 can be further divided into at least three genotypes PCV2a, PCV2b and PCV2c. Prior to 2003, PCV2a was the predominant strain in swine herds and all current commercial vaccines are based on this genotype. However, PCV2b has now become the main genotype circulating throughout the world's swine herds (Meng, 2012). PCV2c has been reported only from a few non-diseased

herds in Denmark (Segales et al., 2008). Although PCV2 is the essential agent in PCVAD, the development of clinical diseases typically requires co-infection with other bacterial or viral swine pathogens. Perhaps the most prevalent co-infecting agent is the porcine reproductive and respiratory syndrome virus (PRRSV) with prevalence as high as 83% in case studies of PCVAD (Opriessnig and Halbur, 2012). PRRSV is an enveloped, single-stranded, RNA virus in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (Chand et al., 2012). Another co-infecting agent commonly isolated in PCVAD field cases is the porcine parvovirus type 1 (PPV1), which is a linear, single-stranded, negative-sense, non-enveloped DNA virus of the family *Parvoviridae* (Opriessnig and Halbur, 2012). The specific mechanisms of co-infection that lead to clinical diseases are not fully understood, but immune modulation by the co-infecting pathogens is thought to play a critical role. In this study we test the hypothesis that the induction of regulatory T-cells (Tregs) contribute to host immune modulations leading to the development of clinical PCVAD, and that vaccination against PCV2 prevents these immunomodulatory effects.

Two types of regulatory T-cells exist in swine, naturally occurring Tregs with a CD25+FoxP3+ phenotype, and inducible Tregs that exist in the periphery and have no common phenotype (Kaser et al., 2012). For the purposes of this study we will be evaluating percentages of Tregs with the CD25+FoxP3+ phenotype. Tregs have a known ability to play a suppressive role in immunity that is achieved by several mechanisms, including the production of immuno-suppressive cytokines IL-10 and TGFβ(Kaser et al., 2012). Previous experiments have shown that PRRSV and PCV2 induce Tregs *in vitro* (Cecere et al., 2012; Silva-Campa et al., 2009)and that PRRSV induces Tregs in vivo*(LeRoith et al., 2011; Silva-Campa et al., 2012)*. The objective of this study was to determine if a combination of viruses associated with PCVAD, including PRRSV, PCV2a and 2b, and PPV1, contribute to Treg induction and production of

immunosuppressive cytokines. To accomplish this objective we analyzed Treg percentages in total peripheral blood mononuclear cell (PBMC) populations, circulating IL-10, TGFβ, and IFN-γ levels in serum, tissue levels of IL-10, TGFβ, and IFN-γ mRNA, and PBMC proliferation from pigs vaccinated against PCV2 followed by a triple challenge with a combination of PRRSV, PPV1 and PCV2a/2b.

Viruses

PCV2a isolate ISU-40895 (Fenaux et al., 2000), and PCV2b isolate NC-16845 (Opriessnig et al., 2008) were propagated in PCV-free PK-15 cells and used in this experiment at an infectious titer of $10^{4.5}$ $TCID_{50}$ per ml. PRRSV isolate ATCC VR2385 (Meng et al., 1994) was propagated in MARC-145 cells and used at an infectious titer of $10^{5.0}$ $TCID_{50}$ per ml. PPV1 isolate NADL-8 was isolated in fetal porcine kidney cells from a naturally infected pig in 1977 and was subsequently passaged in fetuses in the pregnant sow model (Opriessnig et al., 2012)and used at an approximate infectious titer of $10^{4.9}$ $TCID_{50}$ per ml. At challenge, each pig received 5 ml of PCV2a, 5 ml of PCV2b, 2.5 ml PRRSV, and 1.0 ml PPV intranasally according to treatment group.

Experimental design

The experimental design for this study has been described elsewhere (Opriessnig et al., 2012). Briefly, 39 specific-pathogen-free (SPF) pigs were separated into 5 treatment groups described by Opriessnig et al., 2012 and shown in table 1. Pigs in the two vaccinated groups received 1 ml of the corresponding PCV2 vaccine (Beach et al., 2010; Fenaux et al., 2004) intramuscularly in the right neck on day -28 post viral challenge (DPC). All pigs except the negative controls were challenged with a combination of PRRSV, PCV2a/2b and PPV1 on DPC

0. Whole blood was collected in heparinized tubes on DPC -28, 0, 7, 14, and 21. Spleen, lung, and tracheobronchial lymph node tissue samples were collected at necropsy which occurred in all pigs on DPC 21.

Isolation of PBMC's and lymphocytes

Heparinized whole blood collected on DPC -28, 0, 7, 14, and 21 was diluted 1:2 with sterile PBS, overlaid on Ficoll-PaqueTM (GE Healthcare, Piscataway, NJ) and used to isolate PBMC's as previously described (Silva-Campa et al., 2009)

Flow cytometry analysis

Flow cytometry analysis was carried out on blood collected on DPC -28, 0, 14, and 21. Whole blood (3 ml) collected into heparinized Vacutainer tubes (Becton Dickson Inc., Franklin Lakes, NJ) was added to 12 ml ACK Lysis Buffer (8.3 g NH_4Cl, 1.0 g $KHCO_3$, 0.0327 g EDTA in 1L deionized water pH 7.2-7.4; Sigma-Aldrich, St. Louis, MO) and gently mixed by manual rotation for 3 min. Tubes were centrifuged at 300 x g at 4 °C for 5 min. Supernatant was discarded and 1 ml of PBS+1% bovine serum albumin (BSA) was added to the remaining pellet. The pellet was washed three times with 1 ml of PBS with 1% BSA and finally resuspended with 1 ml of PBS+1%BSA. Cells were sequentially stained with mouse anti-porcine CD4 (VMRD, Pullman, WA), goat anti-mouse IgG2b:Alexa-fluor647 (Invitrogen, Carlsbad, CA), mouse anti-porcine CD25 (AbD Serotec, Raleigh, NC), goat anti-mouse IgG:FITC (AbD Serotec, Raleigh, NC), and SPRD conjugated anti-CD8α (Fisher Scientific, clone 76-2-11). For intracellular staining, cells were permeabilized with a FoxP3 permeabilization/fixation buffer kit followed by staining with anti-mouse/rat FoxP3:PE that reacts with porcine FoxP3 (eBioscience Inc., San Diego, CA). An unstained sample was used for autofluorescence control and single color

controls were used for all antibodies. Flow cytometry analysis was conducted using a FACSCalibur cytometer (Becton-Dickinson Biosciences, San Jose, CA) and analyzed using FlowJo 7.6.3 software. PBMCs were gated based on forward and side scatter. Flow cytometry analysis of Treg percentages was determined from all pigs in triplicate samples.

Serum cytokine analysis

Serum samples from blood samples collected on DPC -28, 0, 7, 14, and 21 were harvested following centrifugation at 1200 x g, at 23 °C for 10 min and then frozen at -80 °C until use. The serum levels of IL-10, TGF-β, and IFN-γ were quantified using commercial ELISA kits according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Serum levels of cytokines were determined from all pigs in triplicate samples.

Analysis of cytokine mRNA expressions

Spleen, lymph node, and lung tissues were used for relative quantifications of IL-10, TGF-β, and IFN-γ mRNA by reverse transcription and real-time PCRs. Spleen, lung, and tracheobronchial lymph node tissue samples collected at necropsy at DPC 21 were immediately submerged in 10 volumes of RNAlater (Qiagen), stored at 7 °C for 48 hours, and then frozen at -80 °C until use. Tissue samples were thawed and 100 mg of each tissue from each pig was homogenized in TriReagent (Molecular Research Center, Inc.) according to the manufacturer's instructions. RNA samples were treated with Ambion® DNA-free™ DNase Treatment & Removal Reagents (Invitrogen) according to the manufacturer's instructions and 1µg of resulting RNA was used to synthesize cDNA (Tetro cDNA synthesis kit, Bioline). From each reverse transcription reaction, 2.5 µl of the products were used to assess relative quantities of each specific mRNA of interest in separate PCR reactions in triplicate wells. The SYBR Green PCR

master mix Sensimix (Bioline) was used for all reactions according to the manufacturer's guidelines. All reactions were carried out using an iQ5Cycler PCR machine (BioRad). Quantification was carried out using the delta-delta CT method using GAPDH mRNA as the housekeeping gene for TGF-β and β-actin as the housekeeping gene for IL-10 and IFN-γ. The two different housekeeping genes were needed for compatible melting temperatures for the respective primers. The following programs were used for the GAPDH/ TGF-β samples (95 °C 10min; 40 cycles of 95 °C 15 s, 62.5 °C 30s, 72 °C 15 s) and β-actin/IL-10/ IFN-γ samples (95 °C 3min; 40 cycles of 95 °C 15 s, 65.5 °C 15s, 72 °C 15 s). Melt curve analysis was performed immediately following each run. The same calibrator sample, composed of pooled mRNA samples from all pigs in all treatment groups, was used to compare relative values within and between reactions (96-well plates). The sequences of primers used for the detection of cytokines as well as for detection of GAPDH and β-actin and their GenBank accession numbers of target gene sequences are listed in Table 2. Gene expression was determined from all pigs in triplicate samples.

Cell proliferation

Differences in cell suppression activity among treatment groups were tested indirectly by evaluating proliferation of PBMCs from whole blood collected on DPC -28, 0,7,14, and 21 as previously described (Mosmann, 1983). Briefly, 2×10^5 cells/well were placed into a 96-well round-bottom plate (Fisher Scientific Company) and proliferation was measured in triplicate after stimulation with 10 μg/ml of PHA. Background proliferation was determined with PBMC cultured in complete medium. After incubating for 90 h at 37 °C in 5% CO_2, 20 μl of the cell proliferation reagent (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI) was added to each well, the plates were incubated (37 °C in 5% CO2)

for an additional 4 hours, and then read at 490 nm with a microtiter well plate reader (Tecan Microplate Reader, Tecan Group Ltd. Mannedorf, Switzerland). Cell proliferation was determined from all pigs using triplicate samples.

Statistical analysis

Data were analyzed using the non-parametric Wilcoxon tests. Data analysis was performed using JMP 9.0.0 (SAS Institute Inc. Cary, NC). Differences were considered to be statistically significant where $p<0.05$.

Results

Analysis of percentages of Tregs in total PBMC populations.

Tri-color flow cytometry was used to analyze lymphocyte populations for three distinct subsets of Tregs: $CD4^{+}CD25^{High}FoxP3^{+}$, $CD8^{+}CD25^{High}FoxP3^{+}$, and $CD4^{+}CD8^{+}CD25^{High}FoxP3^{+}$ phenotypes (Fig. 1). 10^4 events were analyzed per triplicate sample. Flow cytometry analysis was performed on DPC -28, 0, 14, and 21. There was a statistically significant increase in the percentage of Tregs in the unvac-PCV2-PRRSV-PPV1 treatment group compared to the control group on DPC 14 in the CD4+ Treg subset, as well as in the CD4+CD8+ Treg subset. There was a statistically significant decrease in the percentages of Tregs in the unvac-PRRSV-PPV1 treatment group versus the control group on DPC 14 in the CD4+ and CD8+ Treg subsets (Fig. 2). The Treg percntages are the porportion of cells expressing $CD25^{High}$ and $FoxP3^{+}$ after gating for CD4 and/or CD8.

Serum levels of cytokines.

Serum cytokine analysis was performed utilizing commercial ELISA kits to detect levels of circulating IFN-γ, TGF-β, and IL-10 on DPC 0, 7, 14, and 21. Statistical analysis revealed lower TGF-β levels in the unvac-PCV2-PRRSV-PPV1 treatment group versus the control group on DPC 7, 14, and 21. Significantly lower levels of TGF-β were also observed in in the unvac-PRRSV-PPV1 treatment group compared to the controls on DPC 7 and 21 (Fig 3A). IL-10 levels were significantly increased in the unvac-PRRSV-PPV1 treatment group on DPC 14 (Fig. 3B). IFN-γ levels were significantly increased in the unvac-PRRSV-PPV1 treatment group versus the controls on DPC 7, and in the unvac-PCV2-PRRSV-PPV1 treatment group on DPC 14, and 21 (Fig. 3C).

Analysis of tissue cytokine mRNA expression.

Spleen, lymph node, and lung tissues collected at necropsy on DPC 21 were analyzed for relative mRNA expression levels of TGF-β, IFN-γ, and IL-10 using RT-PCR techniques. Statistical analysis revealed a statistically significant increase in IL-10 in the lung tissue in the unvac-PCV2-PRRSV-PPV1 treatment group versus the control (Fig. 4).

Cell proliferation

No statistically significant difference in cell proliferation in response to PHA was found between treatment groups.

Discussion

Pigs in the unvac-PCV2-PRRSV-PPV1 treatment group developed clinical manifestations of PCVAD, including; significantly higher lesion scores, and increased PCV2 viremia compared to the controls (Opriessnig et al., 2012). Serology results, detection and

quantification of viral nucleic acid in infected pigs, and lesion scores are reported elsewhere (Opriessnig et al., 2012). Among the vaccinated treatment groups no statistically significant changes in cytokine expression or Treg percentages was found in any of the experimental tests performed compared to the controls. These findings suggest that both the PCV2a genotype vaccine and the PCV2b genotype vaccine induce similar host immune responses. This corresponds with the findings in the concurrent vaccine study that both the PCV2a and PCV2b genotype vaccines provide protection against the development of clinical PCVAD (Opriessnig et al., 2012).

In the unvac-PCV2-PRRSV-PPV1 treatment group, an increase in the percentage of Tregs was observed in two of the three Treg subsets at DPC 14, along with an increase in relative expression of IL-10 mRNA in the lung tissue collected at necropsy. The increase in this treatment group indicates that the addition of PCV2 to the viral challenge does induce Treg differentiation. However, no corresponding *in vitro* cell suppression, or increase in circulating IL-10 or TGF-β levels were seen in this treatment group. The upregulation of IFN-γ observed on DPC 14, and 21 indicates a more typical cell mediated response to viral challenge as the infection proceeds in this treatment group. Previous studies have found that co-infections with PCV2 and PRRSV increase IL-10 expression while suppressing IFN-γ production (Shi et al., 2010). In the current experimental model upregulation of IL-10 was observed only locally, as IL-10 mRNA in the lung tissue, with no increase of circulating serum IL-10 levels. Our experimental model also found no decrease in IFN-γ production, but rather a significant increase in serum IFN-γ levels. There are several possible reasons for these conflicting results. First, the addition of PPV1 to the viral challenge may confound the observed immune modulation. Previous reports of immune modulation in humans by parvovirus demonstrated the activation of

STAT3, and possible inhibition of STAT1 (Duechting et al., 2008). The same type of immune modulation could be occurring with PPV1 infections leading to an altered cytokine response. Second, strain dependent immune modulation by PPRSV has also been demonstrated in the literature, specifically the ability of different PRRSV strains to induce Tregs (Silva-Campa et al., 2010).

Production of IL-10 and TGF-β is an important mechanism of Treg mediated immune suppression that can potentiate viral persistence in the host. However, we were unable to demonstrate increased IL-10 and TGF-β production in this study, although we were able to show an increase in Treg percentages. Our findings suggest that other mechanisms of Treg mediated immune suppression may also be involved, such as, the downregulation of co-stimulatory molecules on APC's (LeRoith et al., 2012), suppression of effector cells by IL-2 consumption, suppression via expression of effector T cell specific transcription factors, rapid suppression of TCR-induced calcium production, and suppression via cAMP, ICER, and NFAT (Schmidt et al., 2012). The specific mechanisms by which Tregs exert their immune modulatory effect are unknown, and further studies are needed.

In the unvac-PRRSV-PPV1 treatment group, an increase of serum IL-10 was observed on DPC 14, but no corresponding induction of Tregs, TGF-β, or *in vitro* cell suppression was detected. The increase in IL-10 levels observed in the study support previous reports that PRRSV infection induces this potent immunosuppressive cytokine (Charerntantanakul et al., 2006; Feng et al., 2003; Suradhat and Thanawongnuwech, 2003). Previous studies have reported that PRRSV induces Tregs both in vitro and in vivo *(LeRoith et al., 2011; Silva-Campa et al., 2009; Wongyanin et al., 2010)*. However, in our study we found a decrease in the percentages of Tregs in this treatment group. Because our study found an increase in IL-10 with no corresponding rise

in Treg percentage, this would appear to support the results from a previous study reporting that Treg induction by PRRSV is dependent on TGF-β rather than IL-10 (Silva-Campa et al., 2009). Again, the presence of PPV1 in the viral challenge may alter the typical immune response to PRRSV.

The transient nature of viral induced immune suppression makes the timing of data collection critical for detecting changes in the immune response to certain pathogens. Although the expected increase in regulatory T cell percentages was observed in the unvac-PCV2-PRRSV-PPV1 treatment group, the lack of expected cytokine production suggests that we may have missed the expected corresponding increase in IL-10 and TGF-β production and cell suppression because of our sampling times. The early onset of Treg induction previously observed is hypothesized to result in a temporary period of immune suppression and delayed viral clearance. The immuno suppressive period is proposed to last until roughly day 14 post viral challenge when a decline in Treg percentages gives rise to the pro-inflammatory environment needed for PCV2 replication in proliferating lymphocytes (Nauwynck et al., 2012) The results from this study revealed a peak in the relative Treg percentages in the unvac-PCV2-PRRSV-PPV1 treatment group DPC 14, followed by a subsequent decrease in Treg percentages to basal levels by DPC 21. The observed decline in Treg percentages and simultaneous increase in IFN-γ appear to support the above proposed model as a mechanism contributing to the development of clinical PCVAD. A more comprehensive monitoring of the cellular and cytokine changes could be achieved by increasing the frequency of data collection. In future work this could facilitate a clearer picture of the immune response in pigs to a multiple viral challenge. The results from this study support the hypothesis that PCV2 addition to a multiple viral challenge induces Tregs *in vivo,* and that vaccination against PCV2 abrogates this immunomodulatory effect of the virus.

The observed ability of PCV2 to induce Tregs could be one mechanism of immune suppression that potentiates clinical PCVAD.

Acknowledgements

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Tables

Table 3.1. Experimental design for vaccination and challenge

Group	No. of pigs	Vaccination at -28 day post-challenge ($10^{3.5}$ $TCID_{50}$/pig)	Virus challenge at 0 days post-challenge
vac2a-PCV2-PRRSV-PPV1	8	PCV1-2a	PVC2a, PCV2b, PRRSV, PPV
vac2b-PCV2-PRRSV-PPV1	8	PCV1-2b	PVC2a,PCV2b, PRRSV, PPV
Controls	7	NONE	NONE
Unvac-PCV2-PRRSV-PPV1	8	NONE	PCV2a, PCV2b, PRRSV, PPV
unvac-PRRSV-PPV1	8	NONE	PRRSV, PPV

Table 3.2. Primer sequences used for the detection of cytokine mRNAs

Gene	Accession number	Forward primer (5'–3')	Reverse primer (5'–3')
TGF-β	AF461808	CTACTACGCCAAGGAGGTCAC	GCCCGAGAGAGCAATACAGG
IL-10	NM214123	CCGACTCAACGAAGAAGGCAC AG	CAGGCTGGTTGGGAAGTGGATG
IFN-γ	DQ839398	TTCAGAGCCAAATTGTCTCCTTC	AAGTCATTCAGTTTCCCAGAGC
GAPDH	DQ845173	CATCATCCCTGCTTCTACC	TGCTTCACCACCTTCTTG
β-actin	DQ845171	CTGCGGCATCCACGAAAC	TGTTGGCGTAGAGGTCCTTGC

Figures

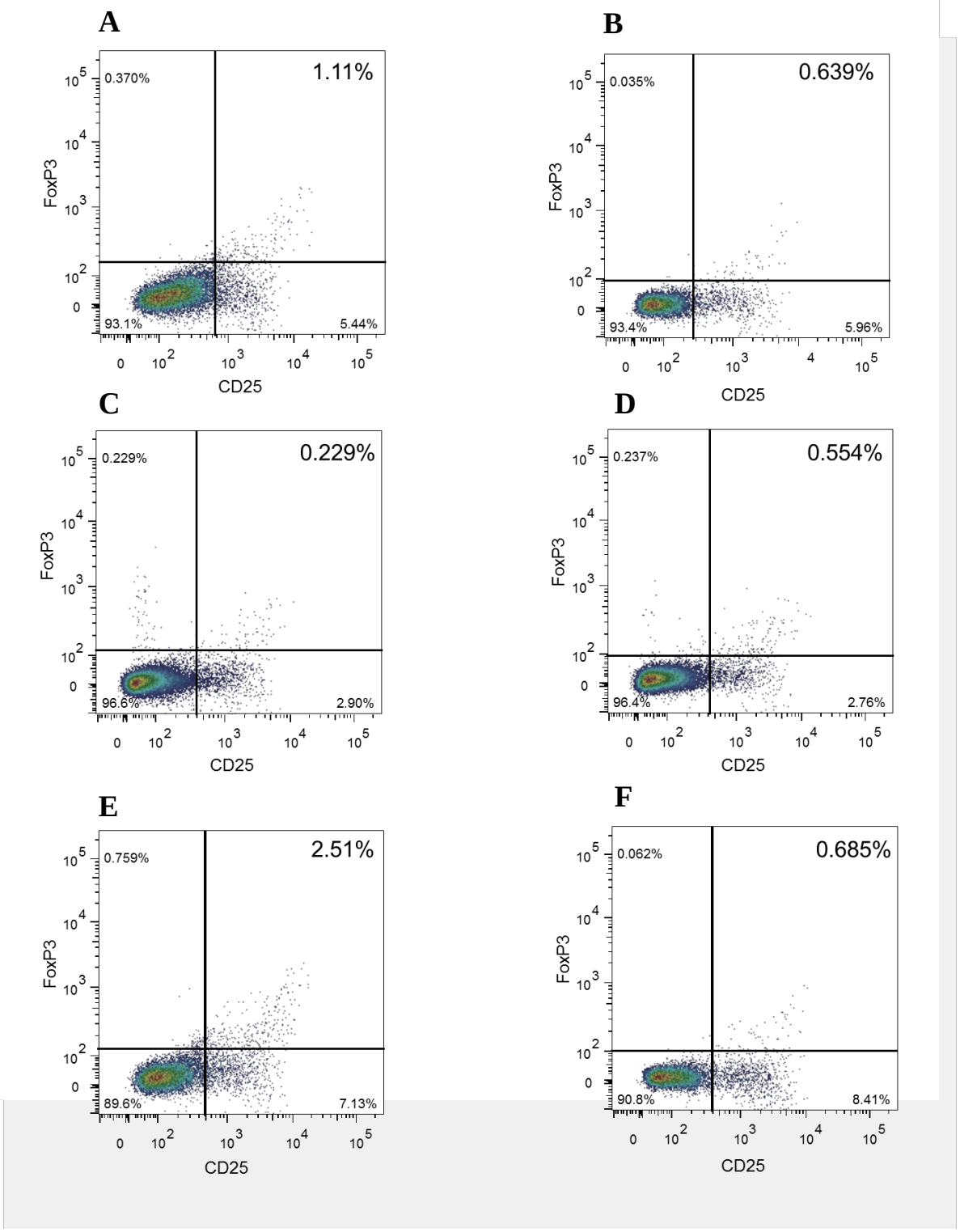
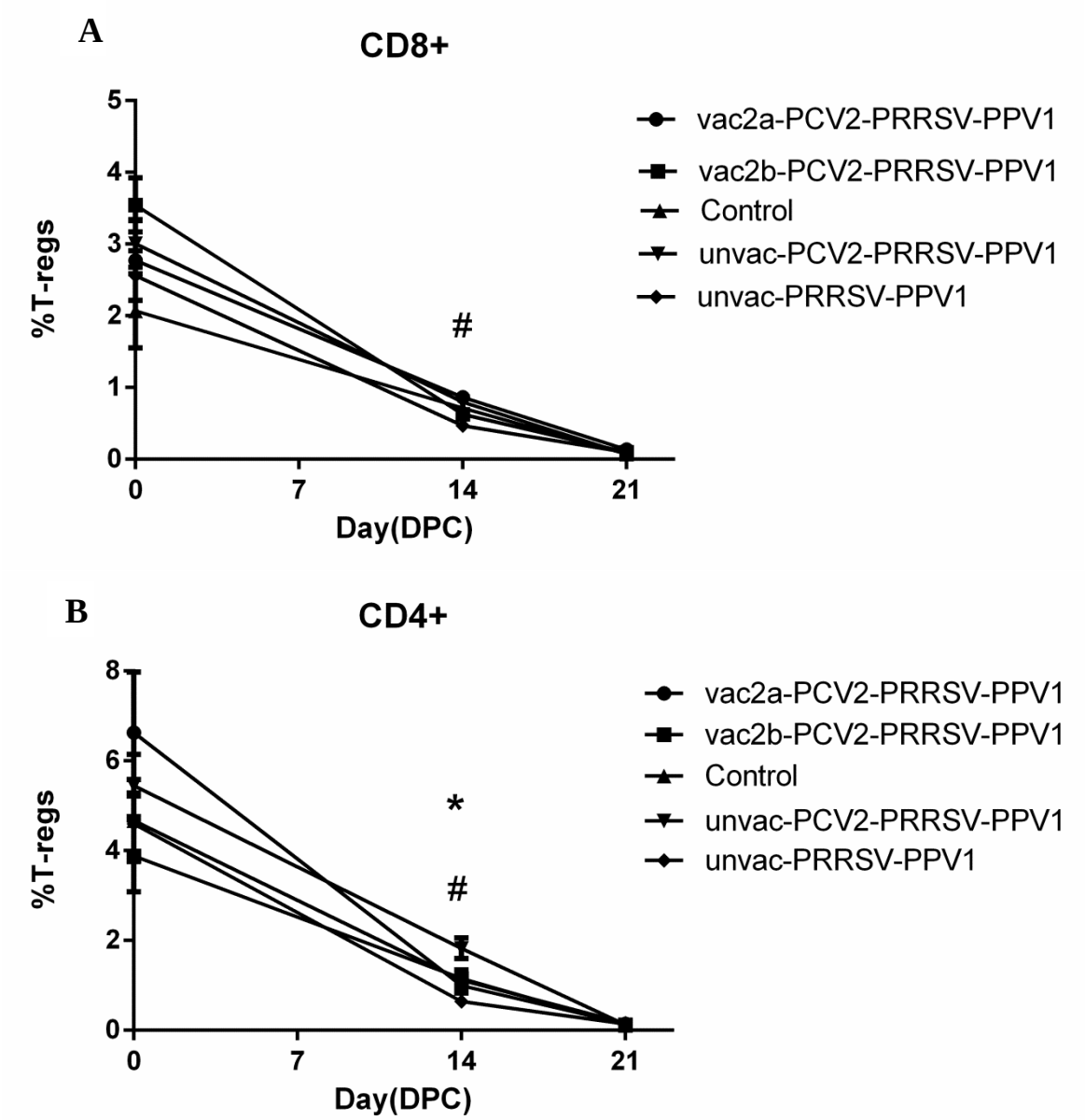

A
0.370%
1.11%
93.1%
5.44%
FoxP3
CD25
B
0.035%
0.639%
93.4%
5.96%
FoxP3
CD25
C
0.229%
0.229%
96.6%
2.90%
FoxP3
CD25
D
0.237%
0.554%
96.4%
2.76%
FoxP3
CD25
E
0.759%
2.51%
89.6%
7.13%
FoxP3
CD25
F
0.062%
0.685%
90.8%
8.41%
FoxP3
CD25

Fig. 3.1 Representative flow cytometry profile of Regulatory T-Cells. (A) unvac-PCV2-PRRSV-PPV1 treatment group $CD4^+$ gated lymphocytes expressing $CD25^+$ and $FoxP3^+$; (B) Control group $CD4^+$ gated lymphocytes expressing $CD25^+$ and $FoxP3^+$; (C) unvac-PRRSV-PPV1 treatment group $CD8^+$ gated lymphocytes expressing $CD25^+$ and $FoxP3^+$; (D) Control group $CD8^+$ gated lymphocytes expressing $CD25^+$ and $FoxP3^+$; (E) unvac-PCV2-PRRSV-PPV1 treatment group $CD4^+CD8^+$ gated lymphocytes expressing $CD25^+$ and $FoxP3^+$; (F) Control group $CD4^+CD8^+$ gated lymphocytes expressing $CD25^+$ and $FoxP3^+$.


A
CD8+
%T-regs
Day(DPC)
0
7
14
21
#
vac2a-PCV2-PRRSV-PPV1
vac2b-PCV2-PRRSV-PPV1
Control
unvac-PCV2-PRRSV-PPV1
unvac-PRRSV-PPV1
B
CD4+
%T-regs
Day(DPC)
*
#
vac2a-PCV2-PRRSV-PPV1
vac2b-PCV2-PRRSV-PPV1
Control
unvac-PCV2-PRRSV-PPV1
unvac-PRRSV-PPV1

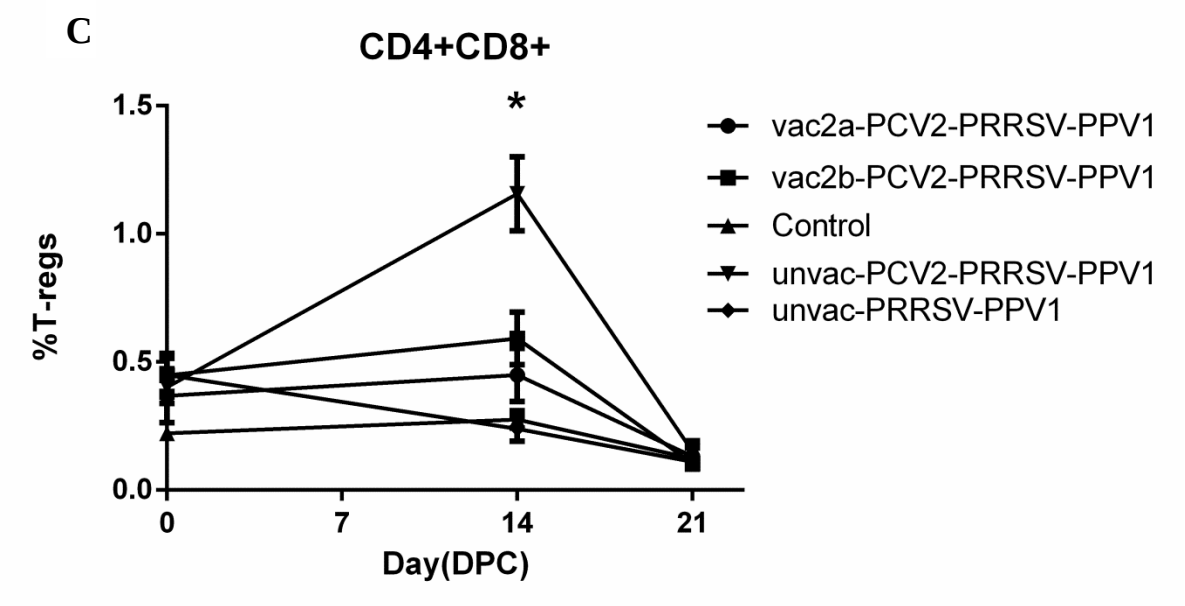
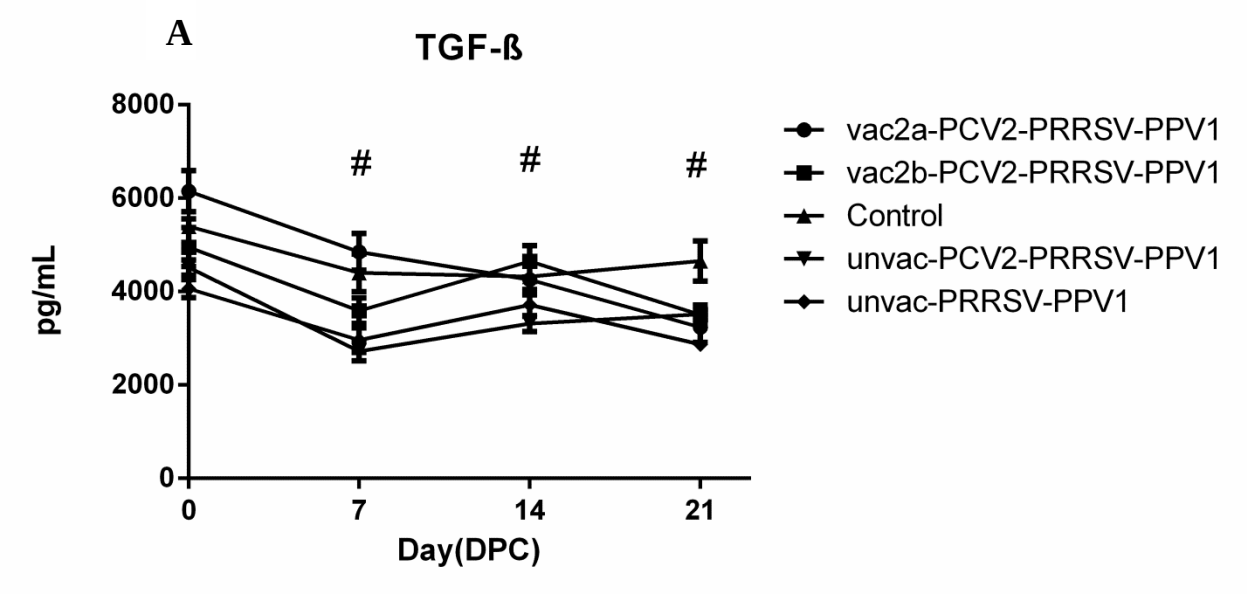
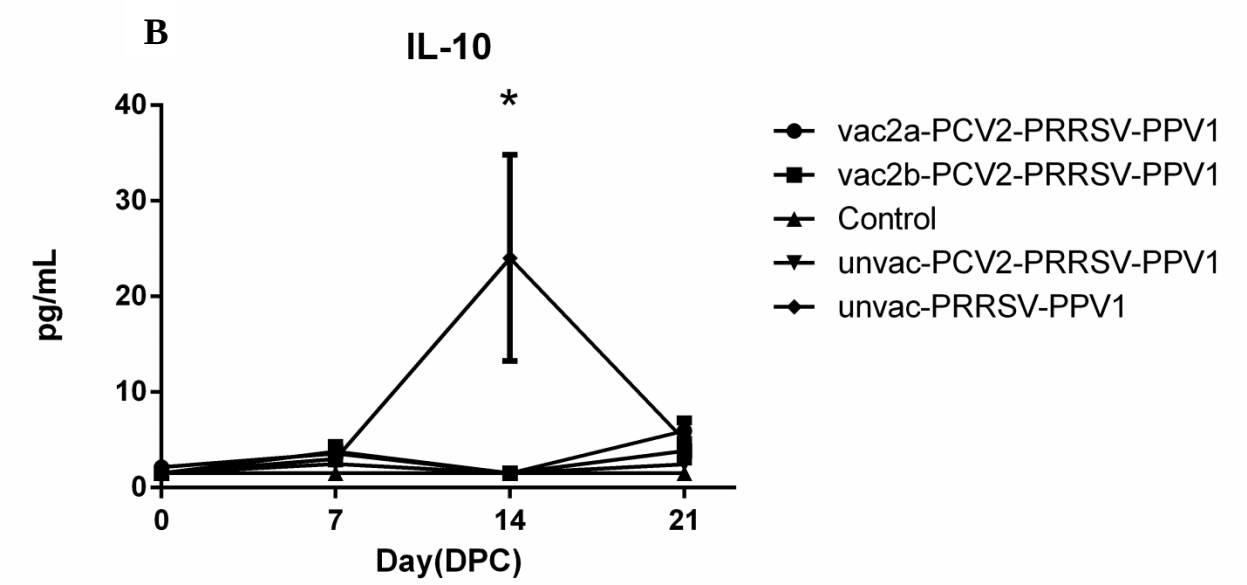


Fig. 3.2 Mean % of regulatory T-cells among different treatment groups over time. (A) $CD4^{+}CD8^{-}CD25^{+}FoxP3^{+}$ T-cells. *Indicates significantly higher % ($p<0.05$) in the unvac-PCV2-PRRSV-PPV1 treatment group versus the control. # Indicates significantly lower % ($p<0.05$) in the unvac-PRRSV-PPV1 treatment group versus the control. (B) $CD4^{-}CD8^{+}CD25^{+}FoxP3^{+}$ T-cells. # Indicates significantly lower % ($p<0.05$) in the unvac-PRRSV-PPV1 treatment group versus the control. (C) $CD4^{+}CD8^{+}CD25^{+}FoxP3^{+}$ T-cells. * Indicates significantly higher % ($p<0.05$) in the unvac-PCV2-PRRSV-PPV1 treatment group versus the control. Each data point represents the mean of the samples. Error bars indicate standard error.


A
TGF-ß
8000
6000
4000
2000
0
pg/mL
#
#
#
0
7
14
21
Day(DPC)
vac2a-PCV2-PRRSV-PPV1
vac2b-PCV2-PRRSV-PPV1
Control
unvac-PCV2-PRRSV-PPV1
unvac-PRRSV-PPV1


B
IL-10
40
30
20
10
0
pg/mL
*
0
7
14
21
Day(DPC)
vac2a-PCV2-PRRSV-PPV1
vac2b-PCV2-PRRSV-PPV1
Control
unvac-PCV2-PRRSV-PPV1
unvac-PRRSV-PPV1

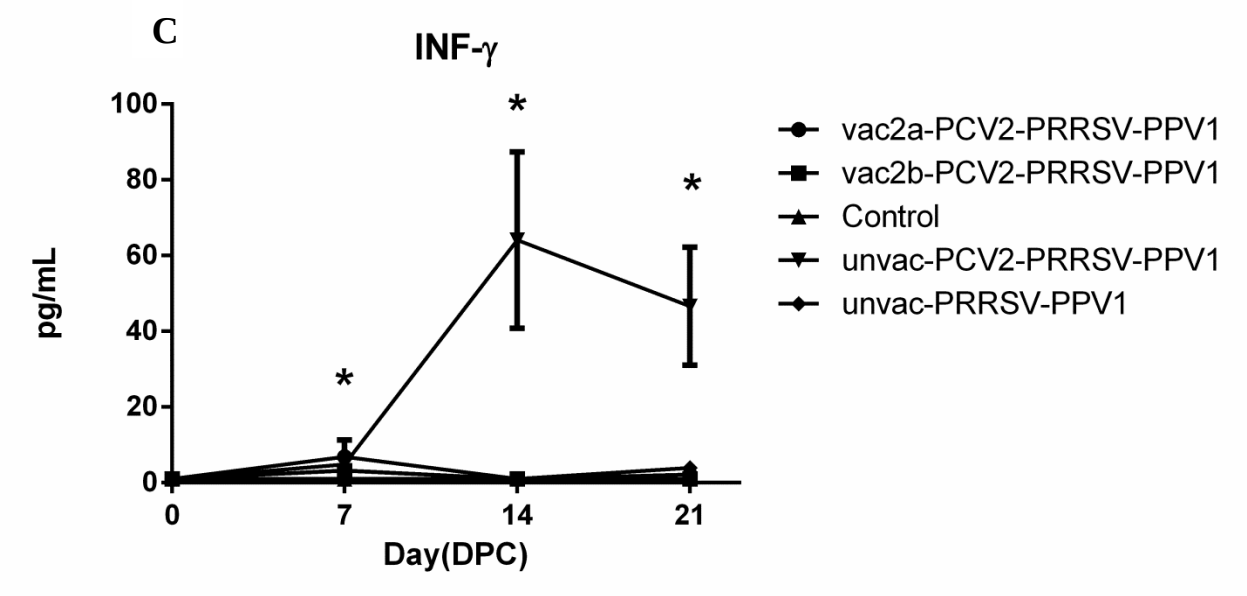


Fig. 3.3 Circulating serum cytokine levels (pg/ml) over time. (A) TGF-β serum levels. # Indicates significantly lower levels ($p<0.05$) in the unvac-PCV2-PRRSV-PPV1 treatment group versus the control on DPC 7, 14, and 21 and lower levels in the unvac-PRRSV-PPV1 treatment group versus the control on DPC 7, and 21. (B) IL-10 serum levels. *Indicates significantly higher levels ($p<0.05$) in the unvac-PRRSV-PPV1 treatment group versus the control on DPC 14. (C) IFN-γ serum levels. * Indicates significantly higher levels ($p<0.05$) in the unvac-PRRSV-PPV1 treatment group versus the control on DPC 7 and in the unvac-PCV2-PRRSV-PPV1 treatment group versus the control on DPV DPC 14 and 21. Each data point represents the mean of the samples. Error bars indicate standard error.

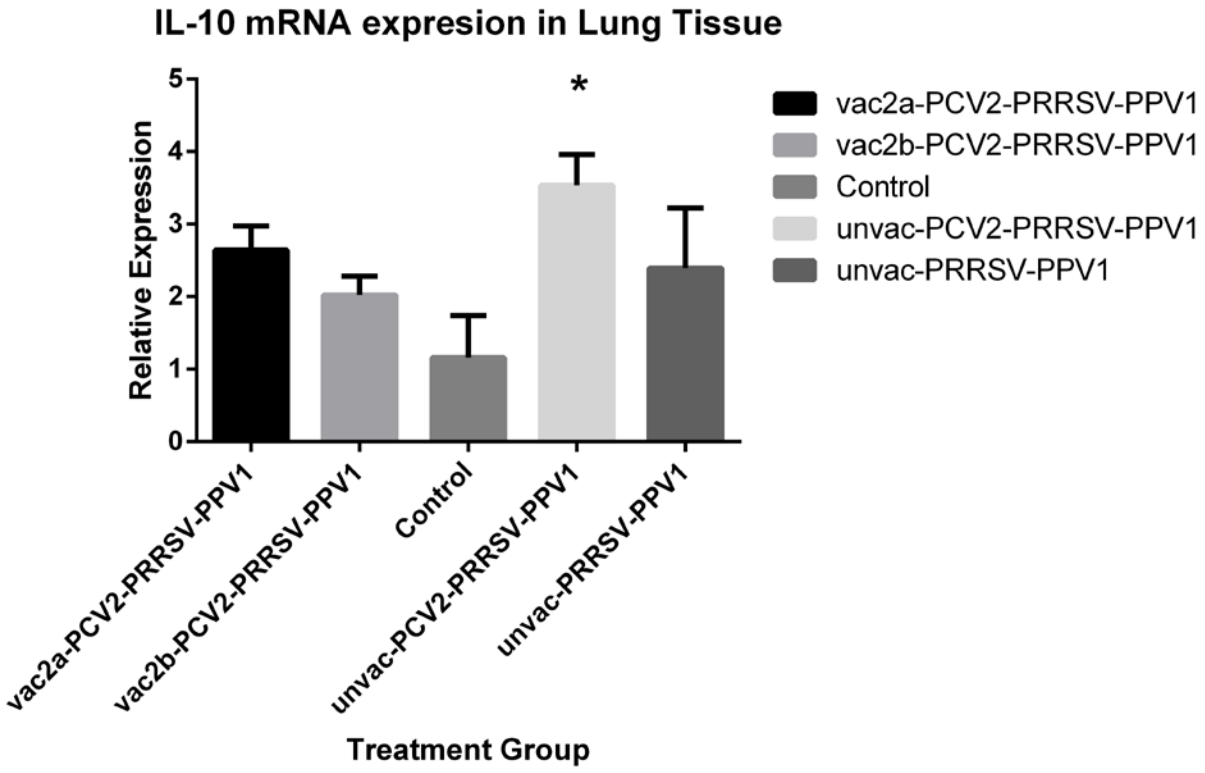


Fig. 3.4 Levels of IL-10 mRNA expression in the lung tissue. * Indicates significantly higher relative expression ($p<0.05$) in the unvac-PCV2-PRRSV-PPV1 treatment group versus the control. Each bar represents the mean of the samples. Error bars indicate standard error.

Chapter 4: PD-L1 expression is increased in monocyte derived dendritic cells in response to porcine circovirus type2 and porcine reproductive and respiratory syndrome virus infections

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Key words

porcine circovirus type 2 (PCV2); porcine circovirus associated disease (PCVAD); porcine reproductive and respiratory syndrome virus (PRRSV); co-infection; programmed death ligand-1 (PD-L1); monocyte derived dendritic cells (MoDC).

Abstract

Host immune system suppression is thought to be crucial in the development of porcine circovirus associated diseases (PCVAD). Many immune suppressive mechanisms have been studied in cases of PCVAD, however, the role of programmed death ligand-1 (PD-L1) during porcine circovirus type 2 (PCV2) infection and PCVAD development has yet to be determined. PD-L1 has become an important research target because of its ability to interfere with effective T-cell activity and proliferation during the course of an immune response. In this study, porcine monocyte derived dendritic cells (MoDC) were infected with different combinations of PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV) and evaluated for expression levels of PD-L1, as well as the expression levels of swine leukocyte antigen 1 and 2 (SLA-1 and SLA-2) as a measure of MoDC stimulatory capacity. PD-L1 expression levels were also tested in

MoDCs after treatment with interferon alpha (IFN-α) and beta (IFN-β). The results showed that the expression levels of PD-L1 were increased in PCV2-infected MoDCs, as well as in PCV2 and PRRSV co-infected MoDCs. The MoDCs infected with PRRSV only also showed a strain-dependent increase in PD-L1 expression. Both IFN-α and IFN-β treatment also increased the expression levels of PD-L1 in MoDCs. SLA-1 and 2 expression levels were increased by PCV2 infection, and altered in the PRRSV, and PCV2/PRRSV co-infected MoDCs in a strain-dependent manner. These results indicate a potential immuno-suppressive role for dendritic cells during PCV2 infection and the development of PCVAD and will be helpful in more fully elucidating the underlying mechanisms leading to clinical PCVAD.

Introduction

Porcine circovirus associated disease (PCVAD) continues to be a major economic problem for swine producing nations throughout the world (Meng, 2012). The essential and causative agent of PCVAD is porcine circovirus type2 (PCV2). Although PCV2 is necessary for the development of PCVAD, co-infection by another pathogen is typically required for full manifestation of the disease (Opriessnig and Halbur, 2012). Despite advances in the prevention of PCVAD through effective vaccination (Beach and Meng, 2012), the mechanisms of co-infection that lead to the development of PCVAD are not fully understood, though immune modulation by PCV2 or a co-pathogen are suspected to play a role (Opriessnig and Halbur, 2012). One of the most prevalent co-infecting pathogens is porcine reproductive and respiratory syndrome virus (PRRSV) (Wellenberg et al., 2004). Both PCV2 and PRRSV have previously been shown to be modulators of the porcine immune system (Darwich et al., 2010; Darwich and Mateu, 2012). However, the potential role of the immune regulatory molecule programmed death ligand-1 (PD-L1) during infection by either virus has yet to be determined.

PD-L1, also known as cluster of differentiation 274, and B7-H1, is a type 1 transmembrane protein with immune suppressing activity (Freeman et al., 2000; Jeon et al., 2007). PD-L1 and its ligand, programmed death-1 (PD-1), have emerged as important research and therapeutic targets because of their role in modulating disease through immune suppression. Increased PD-L1 expression on antigen presenting cells (APCs) has been shown to aid in virus survival and decrease T-cell activity (Meier et al., 2008). The effect of PCV2 infection on PD-L1 expression is currently unknown, but increases could be one underlying mechanism of immune suppression leading to clinical PCVAD.

In this study we examined the potential role of PD-L1 in PCV2 and/or PRRSV infections by evaluating PD-L1 expression levels in porcine monocyte derived dendritic cells (MoDCs) infected with different combinations of viruses. We also examined the effect of interferon alpha (IFN-α) and beta (IFN-β) treatments on porcine MoDC PD-L1 expression levels. Here we found that infection of MoDC by PCV2 and/or PRRSV increased PD-L1 expression and altered the expression of swine leukocyte antigen 1 and 2 (SLA-1 and SLA-2) in a strain-dependent manner. We also found that IFN-α and IFN-β treatment increased MoDC PD-L1 expression. These findings suggest that increased APC PD-L1 expression may be a previously unknown mechanism of immune suppression in PCV2 infections and thus the development of PCVAD.

Materials and Methods

Viruses

The PCV2b isolate used for this experiment was a virulent strain NC-16845 (Fenaux et al., 2000). Two PRRSV wild-type strains and one PRRSV vaccine strain were utilized in this experiment. The wild type PRRSV strains were VR-2385 (Meng et al., 1994), and NADC-20

(kindly provided by Dr. Kelly Lager of National Animal Disease Center, Ames, Iowa). The vaccine strain was a modified live vaccine (MLV) from Ingelvac (Boehringer Ingelheim, St. Joseph Mo.). All viruses were used at a multiplicity of infection (MOI) of 1 according to the treatment groups listed in Table 1.

Cytokines

To evaluate the role of Type-1 IFNs on porcine PD-L1 induction, MoDCs were treated with porcine IFN-α (PBL Assay Science, Piscataway, NJ), porcine IFN-β (Abcam, Cambridge, MA.) or a combination of porcine IFN-α and IFN-β. Cytokine treatment groups are listed in Table 2.

Cell isolation and cell culture

Heparinized whole blood was collected from specific-pathogen-free (SPF) pigs that were confirmed to be free of PCV2 and PRRSV by PCR and ELISA. Twelve animals were used in this study in accordance with the Virginia Tech Institutional Animal Care and Use Committee guidelines. Whole blood was diluted 1:2 with sterile PBS, overlaid on Ficoll-PaqueTM (GE Healthcare, Piscataway, NJ) and used to isolate peripheral blood mononuclear cells (PBMC) as previously described (Silva-Campa et al., 2009). Porcine MoDCs were generated from the PBMC population using the method previously described (Cecere et al., 2012). Briefly, PBMCs were plated in T25 tissue culture flasks and incubated overnight in complete medium at 37 °C with 5% CO_2 to allow monocyte adherence. Non-adherent cells were removed and the flask washed twice with complete media. The remaining monocytes were cultured at 37 °C with 5% CO_2 in complete medium supplemented with 20 ng/ml recombinant porcine IL-4 and 20 ng/ml

recombinant porcine GM-CSF. After six days, MoDCs were washed in complete media and exposed to their corresponding cytokine or viral treatments for 24 hours.

Flow cytometry analysis

Flow cytometry analysis of PD-L1 surface expression was performed using FACSAria cytometer (Becton-Dickinson Biosciences, San Jose, CA). PD-L1 expression was evaluated using a polyclonal, CY-5 conjugated, rabbit anti-porcine PD-L1 antibody (Bioss, Woburn, MA.). SLA-1 expression was evaluated using a monoclonal, FITC conjugated, mouse anti-porcine SLA-1 antibody (AbD Serotec, Raleigh, NC.) SLA-2 expression was evaluated using a FITC conjugated, mouse anti-human MHC II monoclonal antibody with porcine cross reactivity (Thermo Scientific, Waltham, Ma.). MoDCs were harvested using Cellstripper™ (Cellgro, Manassas, VA) and centrifuged at 300xg for 10 minutes at 7°C. The resulting pellet was resuspended in BD pharmingen stain buffer (BSA) (BD biosciences, San Diego, CA.) for antibody staining. All staining was carried out for 30 minutes in the dark. Median fluorescent intensity (MFI) of each treatment group was used to determine surface expression levels. All samples were tested in triplicate.

Analysis of mRNA expression

Total mRNA were isolated from MoDCs using TriReagent (Molecular Research Center, Inc.) according to the manufacturer's instructions for adherent cells. RNA samples were treated with Ambion® DNA-free™ DNase Treatment & Removal Reagents (Invitrogen) according to the manufacturer's instructions. Resulting RNA was then reverse transcribed to cDNA using a Tetro cDNA synthesis kit according to the manufacturer's instructions (Bioline USA Inc., Taunton, Ma). Relative mRNA expression levels were evaluated using porcine PD-L1, SLA-1,

and SLA-DQA TaqMan® assays (Applied Biosystems®, Life Technologies, Grand Island, NY) according to the manufacturer's instructions. 16s rRNA (Life Technologies) was used as an endogenous control. All reactions were carried out using a 7500 Fast Real Time PCR System (Applied Biosystems). The following program was used for all RT-PCR assays; an initial hold for 2 minutes at 50°C, a 95°C hold for 20 seconds, followed by 40 cycles of 95°C for 1 second, 60°C for 20 seconds. All samples were tested in triplicate.

Statistical analysis

Data analysis was performed using JMP 11.0 (SAS Institute Inc. Cary, NC) using Students t-test. Differences between treatment groups were considered to be statistically significant where $p<0.05$.

Results

Porcine MoDC PD-L1 expression profiles during viral infection in vitro

To test the effects of viral infection on PD-L1 expression, 10^5 MoDCs were infected with the different viral treatment groups listed in Table 1 for 24 hours. The resulting cells were then tested for PD-L1 gene expression and corresponding PD-L1 surface expression. All viral treatment groups had significantly increased PD-L1 gene expression among infected MoDCs with the exception of the PRRSV NADC-20 virulent strain and the PRRSV MLV vaccine strain (Fig. 1B). Surface expression of PD-L1 was also significantly increased in the same treatment groups, confirming the gene expression data (Fig. 1C). The presence of PCV2 in co-infected treatment groups appears to enhance the increases observed in the PRRSV-infected groups with the largest PD-L1 expression increase occurring in the PCV2/VR-2385 co-infected treatment group.

Porcine MoDC SLA expression during viral infection

To determine the effect of viral infection on SLA expression, 10^5 MoDCs were infected with the different virus groups listed in Table 1 for 24 hours. The resulting cells were subsequently tested for gene expression levels of SLA-1 and SLA-2. Previous studies found that PRRSV strain CNV-3 downregulated SLA-1 and SLA-2 expression among infected MoDCs (Park et al., 2008). Supporting this previous study, we found that porcine MoDCs infected with PRRSV only had significantly decreased gene and surface expression levels of SLA-1 (Fig. 2A and 2B). MoDCs infected with PCV2 only had a significant increase in SLA-1 expression. The addition of PCV2 in the co-infected treatment groups did not affect the PRRSV-mediated decreased expression of SLA-1 except for the PCV2/PRRSV-MLV co-infected MoDCs. PCV2/PRRSV-MLV co-infected MoDCs had a significant increase in SLA-1 gene and surface expression levels (Fig. 2A and 2B). SLA-2 gene expression is decreased in both individual and co-infected viral treatment groups containing wild-type PRRSV strains, however, statistically significant decreases were only observed in the surface expression levels of SLA-2 (Fig. 3A and 3B). The PCV2-only treatment group had a significant increase in SLA-2 gene and surface expression, as well as the PCV2/PRRSV MLV co-infected treatment group (Fig. 3A and 3B).

Porcine MoDC PD-L1 expression following type 1 interferon treatment

Since type 1 IFN expression can be altered by PRRSV and PCV2, we wanted to determine if type 1 IFNs affect PD-L1 expression levels in swine. 10^5 MoDCs were treated for 24 hours according to the treatment groups listed in Table 2. The MoDCs were then tested for both gene and surface expression levels of PD-L1. The results show that both gene and surface expression levels of PD-L1 are significantly increased among MoDCs treated with IFN-α and

IFN-β, with the largest increase observed in the IFN-α/IFN-β co-treatment group (Figs. 4A and 4B).

Discussion

Increased expression levels of PD-L1 in dendritic cells have previously been shown to negatively impact viral load and T-cell function, thereby decreasing the overall immune response to viral infections (Meier et al., 2008; Pen et al., 2014). In the case of PCV2 infection and PCVAD, immune suppression has long been suspected of playing an important role in the development of clinical disease. This study demonstrates for the first time that increased PD-L1 expression among APCs may be an underlying mechanism of immune suppression in the development of PCVAD during PCV2 infection.

In the PCV2 infection treatment group we observed a significant increase in MoDC PD-L1 expression in addition to a significant increase in MoDC expression levels of SLA-1 and 2. Under these infection conditions, dampened T-cell responses would not be expected since previous studies have found that, during the course of singular PCV2 infection, both CD4+ and CD8+ T-cells can contribute to IFN-γ production and aide in viral clearance (Fort et al., 2009a). The increase in MoDC stimulatory markers SLA-1 and 2 would be expected to result in better T-cell survival and proliferation despite the increase in PD-L1.

In the PRRSV infection groups, there were strain-dependent increases in MoDC PD-L1 expression and alteration of SLA expression levels. In the case of the wild-type PRRSV strain VR-2385, we observed a significant increase in MoDC PD-L1 expression accompanied by a significant decrease in both SLA-1 and SLA-2 expression levels. Conversely, wild type PRRSV strain NADC-20 did not induce an increase in PD-L1 expression, however, decreased SLA-1 and

SLA-2 expression levels were observed. Multiple studies have shown that the cell-mediated immunity (CMI) response to PRRSV is slow and erratic, and occurs with differing effectiveness in a strain-dependent manner (Charerntantanakul et al., 2006; Lopez Fuertes et al., 1999; Meier et al., 2003). Our results suggest that differences in PD-L1 induction may contribute to the unusual CMI responses to PRRSV strains. A previous study found significant decreases in IFN-γ production among T-cell populations incubated with VR-2385 infected MoDCs compared to T-cells incubated with uninfected control MoDCs (Charerntantanakul et al., 2006). The increase in PD-L1 expression along with the decrease in SLA expression observed in this study is a possible explanation for the ineffective T-cell response observed in the previous study. Among PRRSV NADC-20 infected MoDCs, the decrease in SLA expression could lead to an erratic T-cell response even though no induction of MoDC PD-L1 expression was observed. The PRRSV MLV strain had no effect on MoDC PD-L1 or SLA-2 expression levels, but did significantly lower the expression of SLA-1. Despite the observed significant decrease in SLA-1 expression levels in PRRSV MLV- infected MoDCs in the present study, previous reports have found that, under field conditions, PRRSV-specific IFN-γ secreting T-cells do develop after vaccination and their presence correlates with viral clearance or protection (Diaz et al., 2006; Martelli et al., 2009). The observed lack of PD-L1 induction in PRRSV MLV-infected MoDCs is one possible contributing factor in the development of an effective T-cell response following vaccination.

In the co-infection model of PCVAD, perhaps no other pathogen plays a larger role in the development of clinical PCVAD than PRRSV. In a 2004 study, PRRSV was identified in 83% of the PCVAD cases examined (Wellenberg et al., 2004). Although co-infection is required, the exact mechanisms leading to clinical PCVAD have not been established. The results of this study indicate that increased PD-L1 expression in dendritic cells may play an important role. The co-

infection groups evaluated in this study, with the exception of the PCV2/MLV co-infection group, all had significant increases in PD-L1 expression while also decreasing SLA expression in infected MoDCs. This increase in PD-L1 expression along with lowered expression of SLA stimulatory molecules could lead to T-cell dysfunction in the cases of PCVAD in which both PCV2 and PRRSV infections are observed. PD-L1 engagement with PD-1 on effector T-cells is an established immune suppression mechanism which blocks T-cell receptor signaling by inhibiting phosphatidylinositol 3-kinase activation and recruiting SHP-1 and SHP-2 leading to T-cell dysfunction and exhaustion (Chemnitz et al., 2004; Francisco et al., 2010; Jeon et al., 2007). An ineffective T-cell response due to increased PD-L1/PD-1 signaling along with decreased expression of stimulatory surface molecules such as SLA would potentially increase the severity of disease observed in animals with PCVAD.

Depletion of lymphocytes and histiocytic replacement are hallmarks of PCVAD. To date many theories have been proposed to explain the lymphocyte depletion observed in cases of PCVAD, including, cytokine or increased Fas ligand (FasL) activation induced apoptosis, viral induced lysis of lymphocytes or their precursors, and destruction of the lymphoid architecture (Darwich and Mateu, 2012; Darwich et al., 2003b; Ladekjaer-Mikkelsen et al., 2002). However, none of the above proposed mechanisms, or combinations of mechanisms, for lymphocyte depletion in PCVAD have been definitively confirmed as the responsible mechanism(s). Increased PD-L1/PD-1 binding has previously been shown to induce T-cell apoptosis during adenovirus and hepatitis B virus infections (Muhlbauer et al., 2006). Based on the results of our study, we propose that increased PD-L1 expression among APCs may contribute to the mechanism(s) of lymphocytic loss, particularly T-cells, in cases of PCVAD.

The increase of PD-L1 expression induced by IFNs-α and β is in agreement with previous research done in other animal models (Muhlbauer et al., 2006; Teijaro et al., 2013). Although previous studies have shown an inhibition of type 1 IFN production among swine plasmacytoid dendritic cell populations infected with PCV2 (Vincent et al., 2007), other research has found that there is still an increase in plasma levels of IFN-α during the course of PCV2 infections (Fort et al., 2009a). In the case of PRRSV infections, research has shown an inhibition of type-1 IFN production in different cell types (Calzada-Nova et al., 2011; Miller et al., 2004). However, other research into cytokine responses during PRRSV infections has shown that there is either no inhibition or weak inhibition of pDC type-1 IFN production and it occurs in a strain-dependent manner (Baumann et al., 2013). Further research is needed to clarify the role of type-1 IFN induction of PD-L1 during PCV2 infections and the development of PCVAD, but based on the findings of this study it could be a potential mechanism contributing to clinical disease.

Many immune suppression mechanisms have been proposed to potentially play a role during co-infection with PCV2 leading to PCVAD. Altered cytokine responses (Opriessnig and Halbur, 2012), APC dysfunction (Liu et al., 2011), the induction of regulatory T-cells (Cecere et al., 2012), and FasL induced apoptosis (Chang et al., 2007), have all been implicated in the development of PCVAD. This study provides evidence for a novel immune suppression mechanism in the form of increased PD-L1 expression among APCs. Continued research will hopefully shed light on the exact impact of increased APC PD-L1 expression in areas such as T-cell dysfunction, T-cell apoptosis, and the induction of regulatory T-cells. This information could ultimately help researchers to better understand the development of PCVAD during PCV2 infections and help design more targeted therapies.

Acknowledgements

The authors would like to thank Melissa Makris (Virginia-Maryland Regional College of Veterinary Medicine Flow Cytometry Laboratory) for her assistance with the flow cytometry for this study. We also thank Dr. Kelly Lager of the U.S. Department of Agriculture's National Animal Disease Center, Ames, Iowa for kindly providing us the PRRSV NADC-20 strain used in this study.

Tables

Table 4.1 Treatment groups for the evaluation of PD-L1, SLA-1, and SLA-2 expression levels in porcine MoDCs during viral infection.

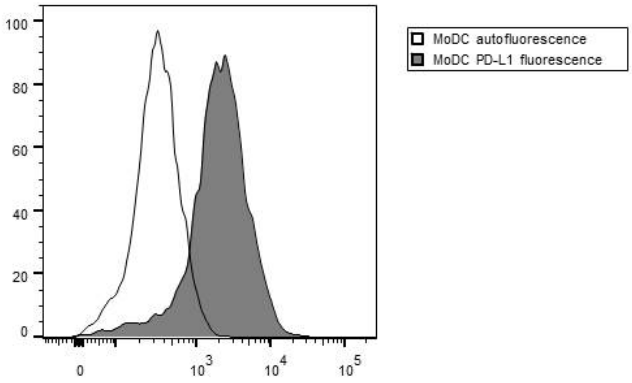
Group Number	PCV2b (MOI 1)	PRRSV (MOI 1)
1 (Control)	**Negative**	**Negative**
2	**NC-16845**	**Negative**
3	**NC-16845**	**VR-2385**
4	**NC-16845**	**NADC-20**
5	**NC-16845**	**MLV**
6	**Negative**	**VR-2385**
7	**Negative**	**NADC-20**
8	**Negative**	**MLV**

Table 4.2 Treatment groups for the analysis of type1 interferon exposure on porcine MoDC PD-L1 expression.

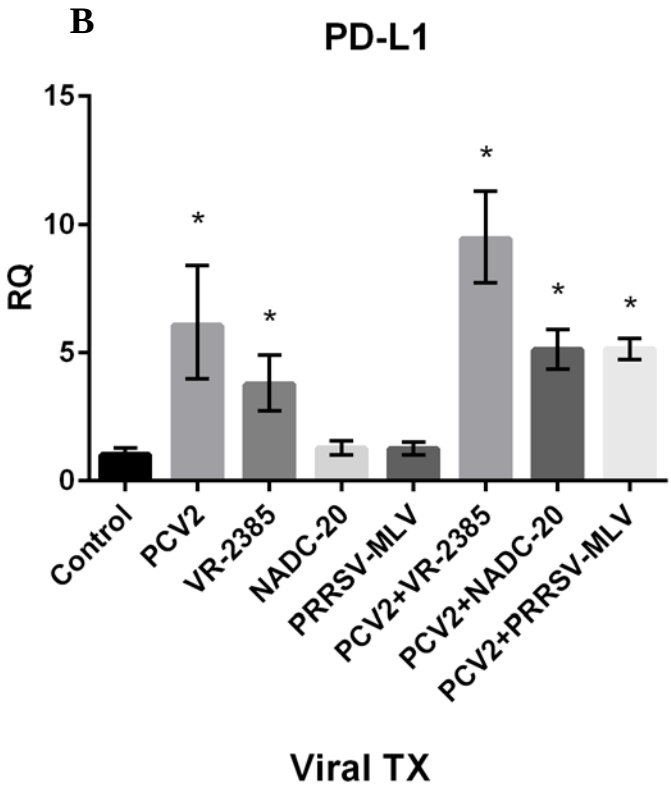
Groups Number	INF-α	IFN-β
1 (Control)	**Negative**	**Negative**
2	**1000 IU**	**Negative**
3	**Negative**	**1000 IU**
4	**500 IU**	**500 IU**

Figures

A


MoDC autofluorescence
MoDC PD-L1 fluorescence
100
80
60
40
20
0
0
10^3
10^4
10^5

B


PD-L1
RQ
15
10
5
0
*
Control
PCV2
VR-2385
NADC-20
PRRSV-MLV
PCV2+VR-2385
PCV2+NADC-20
PCV2+PRRSV-MLV
Viral TX

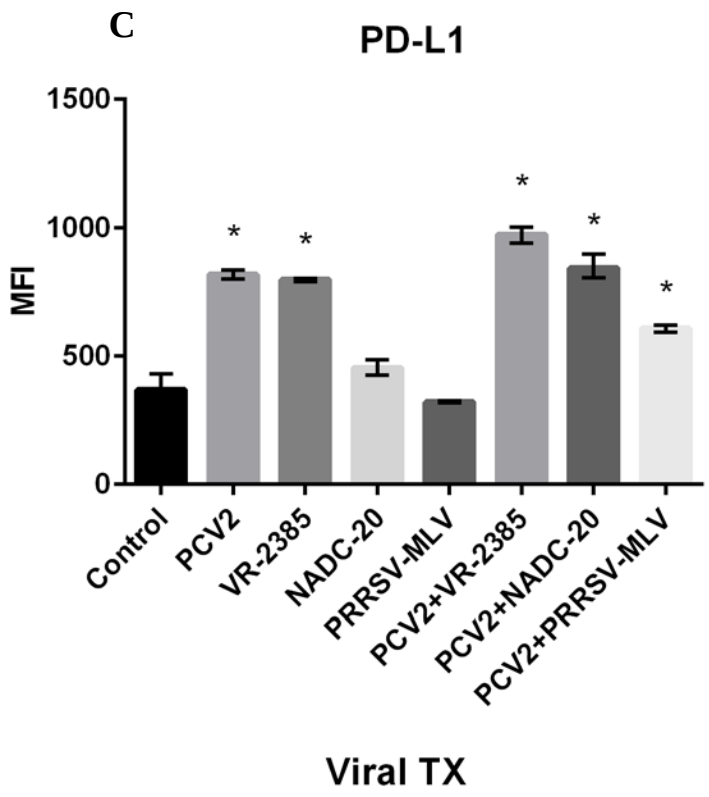
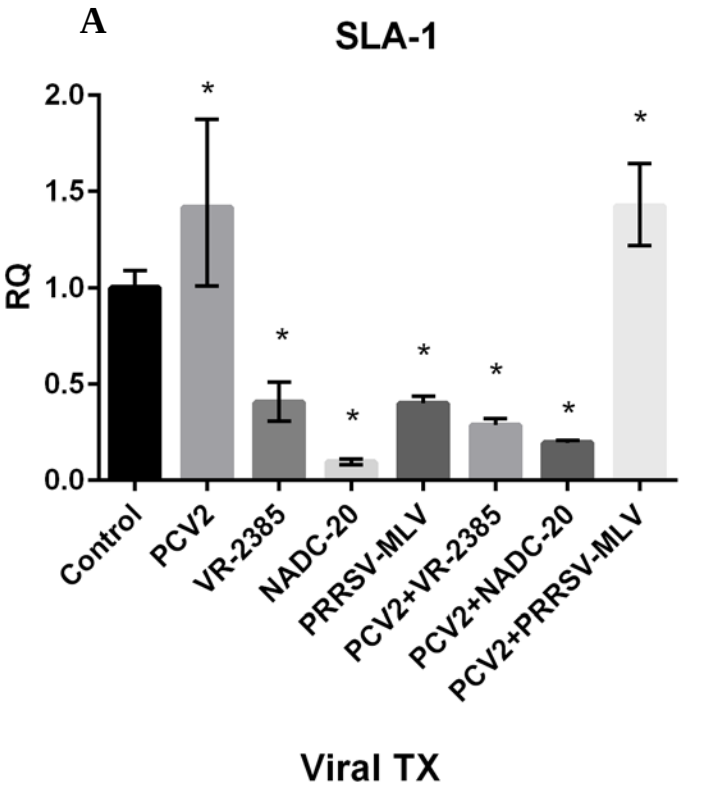


Figure 4.1 A) Representative flow cytometry analysis of PD-L1 MFI increase compared to the autofluorescence control as observed in the PCV2/PRRSV VR-2385 co-infection treatment group**. B)** Relative mRNA transcript levels of PD-L1 in porcine MoDCs after 24 exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of three samples. Error bars indicate standard error. **C)** Porcine MoDC PD-L1 surface expression levels determined by flow cytometry MFI after 24 exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of three samples. Error bars indicate standard error.


A
SLA-1
RQ
2.0
1.5
1.0
0.5
0.0
Control
PCV2
VR-2385
NADC-20
PRRSV-MLV
PCV2+VR-2385
PCV2+NADC-20
PCV2+PRRSV-MLV
Viral TX

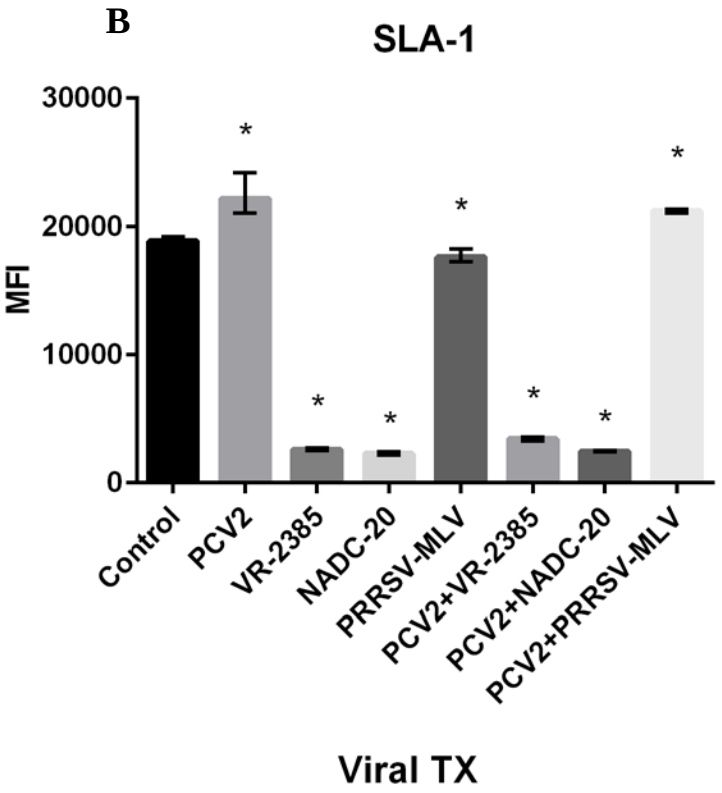
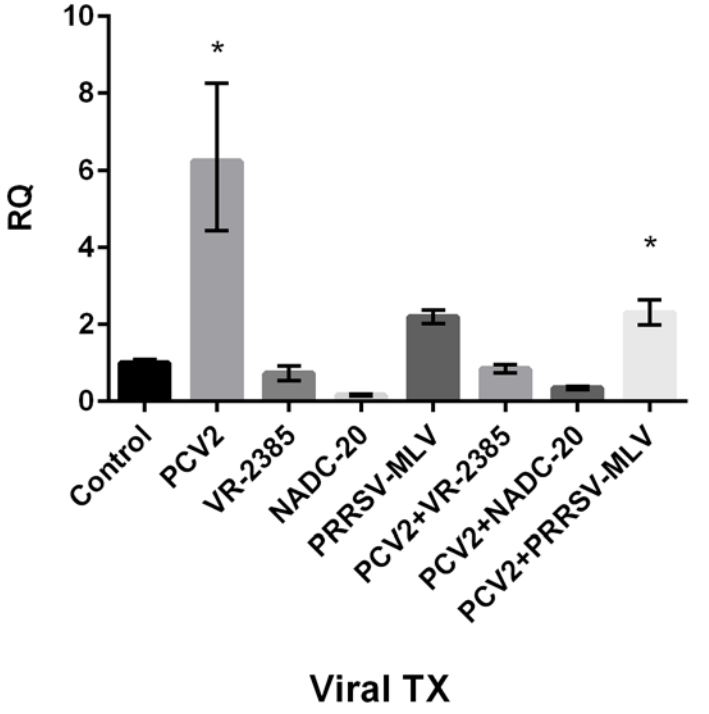

B
SLA-1
MFI
30000
20000
10000
0
Control
PCV2
VR-2385
NADC-20
PRRSV-MLV
PCV2+VR-2385
PCV2+NADC-20
PCV2+PRRSV-MLV
Viral TX

Figure 4.2 A) Relative mRNA transcript levels of SLA-1 in porcine MoDCs after 24 exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of three samples. Error bars indicate standard error. **B)** Porcine MoDC surface expression levels of SLA-1 determined by flow cytometry MFI after 24 exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of three samples. Error bars indicate standard error.

A


SLA-2
RQ
10
8
6
4
2
0
*
*
Control
PCV2
VR-2385
NADC-20
PRRSV-MLV
PCV2+VR-2385
PCV2+NADC-20
PCV2+PRRSV-MLV
Viral TX

B

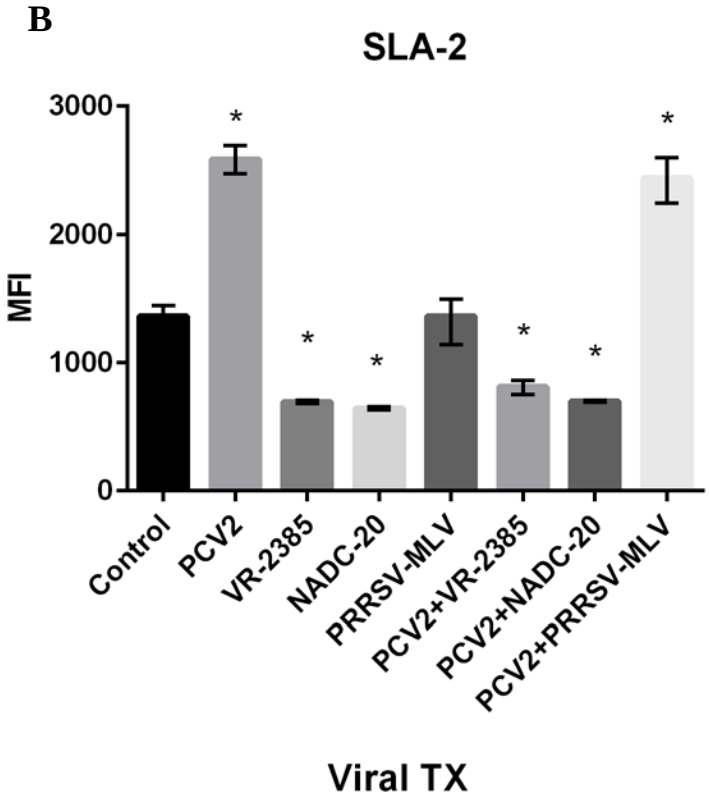

SLA-2
MFI
3000
2000
1000
0
*
*
*
*
*
*
Control
PCV2
VR-2385
NADC-20
PRRSV-MLV
PCV2+VR-2385
PCV2+NADC-20
PCV2+PRRSV-MLV
Viral TX

Figure 4.3 A) Relative mRNA transcript levels of SLA-2 in porcine MoDCs after 24 exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of three samples. Error bars indicate standard error**. B)** Porcine MoDC surface expression levels of SLA-2 determined by flow cytometry MFI after 24 exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of three samples. Error bars indicate standard error.

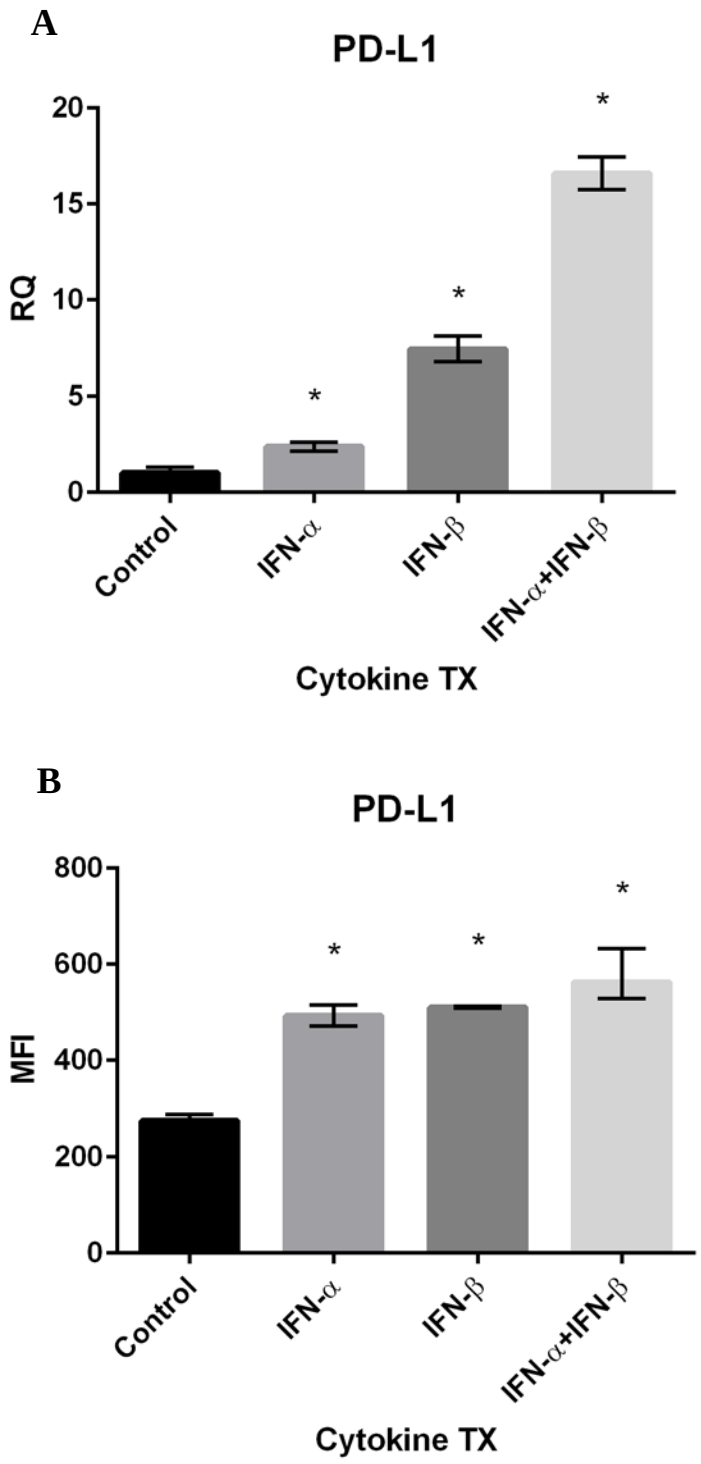


Figure 4.4 A) Relative mRNA transcript levels of PD-L1 in porcine MoDCs after 24 hour treatment with IFN-α and/or IFN-β. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of three samples. Error bars indicate standard error. **B)** Porcine

MoDC surface expression levels of PD-L1 after 24 hour treatment with IFN-α and/or IFN-β determined by flow cytometry MFI. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of three samples. Error bars indicate standard error.

Chapter 5: The PD-L1/CD86 ratio is increased in dendritic cells co-infected with porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus, and the PD-L1/PD-1 axis is associated with anergy, apoptosis, and the induction of regulatory T-cells in porcine lymphocytes

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Key words

porcine circovirus type 2 (PCV2); porcine circovirus associated disease (PCVAD); porcine reproductive and respiratory syndrome virus (PRRSV); Co-infection; Programmed death ligand-1 (PD-L1); programmed cell death-1 (PD-1); monocyte derived dendritic cells (MoDC).

Abstract

Porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) continue to have a negative economic impact on global swine production operations. Host immune modulations that potentiate disease during PCV2 and/or PRRSV infections are important areas of ongoing research. In this study, we evaluated the expression levels of PD-L1, CD86, and IL-10 in order to phenotype dendritic cells following viral infection with PCV2b and/or PRRSV. The results showed that the inhibitory marker PD-L1 was significantly increased in monocyte derived dendritic cells (MoDC) in both singular PCV2

infection and PCV2/PRRSV co-infections. MoDC expression of stimulatory marker CD86 was significantly increased during singular PCV2 infections, while it was significantly decreased in the treatment groups co-infected with both PCV2 and PRRSV. IL-10 production was highest among MoDCs that were co-infected with PCV2 and PRRSV. These results indicate that dendritic cells develop a regulatory phenotype following PCV2/PRRSV co-infections. We further investigated the role of the PD-L1/PD-1 axis in lymphocyte anergy, apoptosis, and the induction of regulatory T-cells in porcine mononuclear cell populations. Lymphocyte populations with normal PD-1 expression had higher percentages of anergic, apoptotic lymphocytes and $CD4^{+}CD25^{HIGH}FoxP3^{+}$ regulatory T-cells when compared to a PD-1 deficient lymphocyte population. These results implicate the PD-L1/PD-1 axis in negative regulation of lymphocyte responses in pigs.

Introduction

Since its discovery in 1998, porcine circovirus type 2 (PCV2) and its associated diseases (PCVAD) have been intensive research topics of interest due to the widespread and negative economic impact they have on the global swine industry (Meng, 2012). Despite advances in PCVAD prevention through awareness and effective vaccination, PCVAD continues to cause economic losses in virtually every swine producing nation (Beach and Meng, 2012). Early research into PCVAD showed that, while PCV2 is associated with PCVAD, it was rarely reproduced by PCV2 infection alone (Allan et al., 1999; Allan et al., 2000; Ellis et al., 1999; Tomas et al., 2008). Co-infection with another swine pathogen, such as porcine reproductive and respiratory syndrome virus (PRRSV), is typically required for the full spectrum of disease manifestation (Beach and Meng, 2012). The exact mechanism(s) of co-infections that lead to PCVAD are still active areas of investigation. However, host immune modulations by PCV2

and/or a co-infecting pathogen are thought to be critical in the development of clinical disease. In particular, suppression of the host immune system has long been suspected of playing a role in the progression of PCVAD. Suppressive immune mechanisms such as virally or cytokine induced apoptosis of lymphocytes, the induction of regulatory T-cells (Tregs), elevated levels of IL-10 production, and altered antigen presenting cell (APC) functionality have all been proposed as contributing to the pathogenesis of PCVAD (Cecere et al., 2012; Darwich et al., 2003a; Darwich and Mateu, 2012; Shibahara et al., 2000). In this study, we further investigated the immune suppression during PCV2 and PRRSV singular infections and co-infections by evaluating IL-10, PD-L1, and CD86 expression in monocyte derived dendritic cells (MoDCs) during viral infection, and the role of the PD-L1/PD-1 axis on lymphocyte responses in swine.

In the first part of this study MoDCs were incubated with different strains of PCV2 and PRRSV and evaluated for gene and protein expression levels of the inhibitory marker PD-L1 and the stimulatory marker CD86. Because PD-L1 and CD86 belong to the same B7 family of proteins (Dong et al., 1999), the balance in the expression of these two molecules can have a major role in determining efficient T-cell responses during viral infections (Shen et al., 2010b). MoDC gene expression levels of IL-10 were also evaluated to further phenotype the MoDCs following viral infection. We hypothesized that there would be an increase in IL-10 and PD-L1 expression and a decrease in CD86 expression among MoDCs co-infected with PCV2 and wild-type strains of PRRSV, thus suggesting a potential inhibitory role for APCs during the development of PCVAD.

Additionally in this study, we evaluated the effect of the PD-L1/PD-1 axis in swine. Previous research in mice has shown that the binding of PD-L1 with PD-1 expressed on T-cells delivers an inhibitory signal that can result in T-cell dysfunction (Kuipers et al., 2006; Martin-

Orozco et al., 2006). Through siRNA silencing of the PD-1 gene in lymphocytes, we hypothesized we would demonstrate a role for the PD-L1/PD-1 axis in either lymphocyte anergy, lymphocyte apoptosis, or the induction of regulatory T-cells, similar to other species (Amarnath et al., 2011; Francisco et al., 2009; Muhlbauer et al., 2006; Shen et al., 2010b), suggesting that increased PD-L1 expression among APCs is a potential mechanism in the pathogenesis of PCVAD.

Materials and methods

Viruses

The PCV2b isolate used for this experiment was a virulent strain NC-16845 isolated from a diseased pig in North Carolina (Fenaux et al., 2000), and the virus was propagated in PCV-free PK-15 cells (Fenaux et al., 2002).Two virulent strains and one vaccine strain of PRRSV were utilized in this experiment. The vaccine strain was a modified live-attenuated vaccine (MLV), Ingelvac PRRS (Boehringer Ingelheim, St. Joseph MO). The virulent PRRSV strains were ATCC VR-2385 (Meng et al., 1994), and NADC-20 (kindly provided by Dr. Kelly Lager of the U.S. Department of Agriculture's National Animal Disease Center, Ames, Iowa). Both virulent PRRSV strains were propagated in MARC-145 cells. The infectious titers of PCV2b NC-16845 as well as PRRSV MLV, NADC-20 and ATCC VR-2385 were determined as described previously (Fang et al., 2006; Fenaux et al., 2002). All viruses were used at a multiplicity of infection (MOI) of 1 according to the treatment groups listed in Table 1.

Cell isolation and cell culture

Heparinized whole blood was collected from specific-pathogen-free (SPF) pigs that were confirmed to be free of PCV2 and PRRSV by PCR and ELISA. Nine animals were used in this

study in accordance with the Virginia Tech Institutional Animal Care and Use Committee guidelines. Whole blood was diluted 1:2 with sterile PBS, overlaid on Ficoll-PaqueTM (GE Healthcare, Piscataway, NJ) and used to isolate peripheral blood mononuclear cells (PBMC) as previously described (Silva-Campa et al., 2009). Porcine MoDCs and lymphocytes were generated from the PBMC population using the method previously described (Cecere et al., 2012). Briefly, PBMCs were plated in T25 tissue culture flasks and incubated overnight in complete medium at 37 °C with 5% CO_2 to allow monocyte adherence. Non-adherent cells were removed by extensive washing and collected for use in the cell proliferation, apoptosis, and Treg assays. The remaining adherent monocytes were cultured at 37 °C with 5% CO_2 in complete medium supplemented with 20 ng/ml recombinant porcine IL-4 and 20 ng/ml recombinant porcine GM-CSF. After six days MoDCs were harvested, washed in complete media and $2.5x10^5$ cells were incubated with their corresponding viral treatments for 24 hours, or utilized in the cell proliferation, apoptosis, and Treg assays.

PD-L1, CD86, and IL-10 expression among virally infected MoDCs

Flow cytometry analysis was performed for surface expression levels of PD-L1 and CD86 among MoDCs from all treatment groups listed in Table 1. Flow cytometry was performed using FACSAria cytometer (Becton-Dickinson Biosciences, San Jose, CA). PD-L1 expression was evaluated using a polyclonal, FITC conjugated, rabbit anti-porcine PD-L1 antibody (Bioss, Woburn, MA.). CD86 surface analysis was evaluated using a polyclonal, FITC conjugated, rabbit anti-porcine CD86 antibody (Bioss). MoDCs were harvested using Cellstripper™ (Cellgro, Manassas, VA) and centrifuged at 300xg for 10 minutes at 7°C. The resulting pellet was resuspended in the BD pharmingen stain buffer (BD biosciences, San Diego, CA.) for antibody staining. All staining was carried out for 30 minutes in the dark. Median

fluorescent intensity (MFI) of each treatment group was used to determine protein surface expression levels. All samples were tested in triplicate.

To evaluate PD-L1, CD86, and IL-10 gene expression levels among MoDCs from the treatment groups listed in Table 1, total mRNA were isolated from MoDCs using TriReagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions for adherent cells. One ug of the resulting RNA was then reverse-transcribed to cDNA using a Tetro cDNA synthesis kit according to the manufacturer's instructions (Bioline USA Inc., Taunton, MA). Relative mRNA expression levels were evaluated using porcine PD-L1, CD86, and IL-10 TaqMan® assays (Life Technologies) according to the manufacturer's instructions. 18s rRNA (Life Technologies) was used as an endogenous control. All reactions were carried out using a 7500 Fast Real Time PCR System (Applied Biosystems, Grand Island, NY). The following program was used for all RT-PCR assays; an initial hold for 2 minutes at 50°C, a 95°C hold for 20 seconds, followed by 40 cycles of 95°C for 1 second, 60°C for 20 seconds. All samples were tested in triplicate.

Electroporation and siRNA

Approximately 10^6 lymphocytes collected from the non-adherent fraction of PBMCs were electroporated in 0.4cm cuvettes (Bio-Rad laboratories, Hercules, CA) using a GenePulser Xcell™(Bio-Rad).Exponential wave form electroporation conditions were as follows; 300V, 350μF capacitance, and 1,000Ω resistance. Experimental groups were electroporated with 100nM of siRNA while the control groups were electroporated with GenePulser® electroporation buffer only (Bio-Rad) at a final volume of 0.3 ml. The PD-1 Stealth RNAi™ siRNA (Life Technologies, Waltham, MA) and the negative control sequence Stealth RNAi™

siRNA (Life Technologies) in this experiment were designed using the BLOCK-iT™ RNAi designer (Life Technologies). PD-1 silencing was measured using a custom porcine PD-1 TaqMan® probe (Life Technologies) (Fig. 4A). A blast search was performed to generate a negative control siRNA to ensure on target silencing of the PD-1 gene (Fig. 4B). The PD-1 siRNA, negative control siRNA, and custom PD-1 TaqMan® probe sequences were designed based on the published sequence for porcine PD-1, GenBank accession number NM_001204379. The sequences of the PD-1 siRNA and negative control siRNA are listed in Table 2. Post electroporation cell viability was routinely found to be between 71% and 89% as determined by Vybrant® MTT cell proliferation assay (Life Technologies).

Lymphocyte proliferation

Following electroporation, lymphocytes were placed in 2ml of complete media supplemented with 10ng/ml of recombinant porcine IL-2 (R&D Systems Inc., Minneapolis, MN) and rested for 24 hours. At 24 hours post-electroporation, the lymphocytes were placed into a T25 flask containing $2.5x10^4$ autologous MoDCs. After 24 hours of co-culture, 1 ml of complete media supplemented with 10ug /ml of PHA-L (Sigma-Aldrich, St. Louis, Mo) was added to the flask. Cell proliferation was measured at 24 hours post PHA-L exposure using a Cell Trace™ violet cell proliferation kit (Life Technologies) according to the manufactures instructions. All samples were tested in triplicate.

Lymphocyte apoptosis

Following electroporation, lymphocytes were placed in 2 ml of complete media supplemented with 10ng /ml of recombinant porcine IL-2 and rested for 24 hours. At 24 hours post-electroporation, the lymphocytes were placed into a T25 flask containing $2.5x10^5$

autologous MoDCs with 5 ml of complete media. Apoptosis was measured at 6, 8, and 12 hours post-MoDC exposure using an annexin V Alexa-Fluor®647 conjugate apoptosis kit (Life Technologies) according to the manufacturer's instructions. DAPI (Life Technologies) was used as a counter stain to detect necrotic cells. All samples were tested in triplicate.

Analysis of Treg percentages

Following electroporation, lymphocytes were placed in 2 ml of complete media supplemented with 10ng/ml of recombinant porcine IL-2 and rested for 24 hours. At 24 hours post-electroporation, the lymphocytes were placed into a T25 flask containing $2.5x10^5$ autologous MoDCs with 5 ml of complete media supplemented with 1 μg of rat anti-porcine CD3 monoclonal antibody (Abcam, Cambridge, MA) and 2ng /ml of porcine TGF-β (R&D Systems) 72 hours post-MoDC exposure, the lymphocytes were removed from the flask, centrifuged at 300xg for 10 minutes. The resulting pellet was washed three times and resuspended with the BD pharmingen stain buffer (BD biosciences). Cells were sequentially stained with FITC-conjugated mouse anti-porcine CD4 (BD biosciences), APC-conjugated rat anti-mouse CD25 with porcine cross-reactivity (Antibodies-Online), and SPRD-conjugated mouse anti-porcine CD8α (Southern Bioscience). For intracellular staining, cells were permeabilized with a FoxP3 permeabilization/fixation buffer kit followed by staining with anti-mouse/rat FoxP3:PE that reacts with porcine FoxP3 (eBioscience Inc., San Diego, CA). The controls for the flow cytometry were an unstained cell population to detect autofluorescence levels, and four cell populations individually stained for CD4, CD8, CD25 and FoxP3. $CD4^+CD25^{HIGH}FoxP3^+$ cell percentages were analyzed using FlowJo® 7.6.3 software. All samples were tested in triplicate.

Statistical analysis

Data analysis was performed using JMP 11.0 (SAS Institute Inc. Cary, NC) using Students t-test. Differences between treatment groups were considered to be statistically significant where $p<0.05$.

Results

Porcine MoDC PD-L1 and CD86 expression during PCV2 and PRRSV infection

In order to evaluate the effect of PCV2 and PRRSV infections on swine MoDC PD-L1 and CD86 expression, $2.5x10^5$ MoDCs were infected with the viral treatment groups listed in Table 1. After 24 hours the MoDCs were analyzed for both gene and surface expression of PD-L1 and CD86. MoDC in all viral treatment groups had significantly increased levels of PD-L1 gene and surface expression levels with the exception of the virulent PRRSV strain NADC-20 and the PRRSV MLV (Fig. 1A and 1B). MoDC CD86 expression was reduced in all MoDC treatment groups containing virulent strains of PRRSV, however, statistically significant decreases were observed only in surface expression levels. The PCV2/MLV co-infection treatment group and the singular PCV2 treatment group both had significant increases in the gene and surface expression levels of CD86 (Fig. 2A and 2B).

IL-10 expression in virally-infected MoDCs

IL-10 is a well-established immunosuppressive cytokine that has been associated with inhibited viral clearance and is proposed to play an important role in the pathogenesis of PCVAD (Darwich and Mateu, 2012; Quintana et al., 2001). To better understand the phenotype of dendritic cell that occurs upon co-infection with PCV2 and PRRSV, the levels of IL-10 gene

expression were tested among the treatment groups listed in Table 1. Significant increases in IL-10 production were observed in MoDCs from the PCV2 only, PCV2/NADC-20, and PCV2/VR-2385 co-infected groups. The NADC-20 only treatment group was the only group with significantly lower expression of IL-10, while the groups containing PRRSV MLV and VR-2385 only groups had no significant change in IL-10 expression. The largest increase in IL-10 gene expression was observed in the PCV2b/VR-2385 co-infected MoDC group (Fig.3).

PD-L1/PD-1 axis effects on lymphocyte apoptosis, proliferation, and Treg percentages

In an attempt to elucidate a potential role for the PD-L1/PD-1 axis in lymphocyte apoptosis, anergy, and the induction of $CD4^{+}CD25^{HIGH}FoxP3^{+}$ regulatory T-cells in pigs, lymphocytes with deficient PD-1 expression ($PD\text{-}1^{(-)}$) were generated and exposed, along with their corresponding treatments, to autologous PD-L1 expressing MoDCs. The $PD\text{-}1^{(-)}$ lymphocyte population showed decreased levels of apoptosis at time points 6, 8, and 12 hours post-MoDC exposure when compared to normal PD-1 expressing lymphocyte populations, however, statistically significant decreases were only observed at 8 and 12 hours post-MoDC exposure (Fig. 5). These results correlate with a previous study in human cells where engagement of the PD-L1/PD-1 axis resulted in increased T-cell apoptosis (Muhlbauer et al., 2006).

The $PD\text{-}1^{(-)}$ lymphocyte population also had a significant decrease in the percentages of non-proliferating cells as compared to normal PD-1 expressing lymphocytes (Fig. 6). The role of the PD-L1/PD-1 axis in T-cell anergy and exhaustion has been established in other animal species (Barber et al., 2006; Selenko-Gebauer et al., 2003; West et al., 2013). This study expands

on these previous studies by experimentally demonstrating the role of the PD-L1/PD-1 axis in lymphocyte anergy in pigs.

$CD4^{+}CD25^{HIGH}FoxP3^{+}$ Treg percentages were found to be increased among normal PD-1 expressing lymphocytes when compared to the $PD\text{-}1^{(-)}$ lymphocytes (Fig. 7). Previous studies in both human and murine species have shown that PD-L1/PD-1 axis can induce and maintain the function of Tregs (Amarnath et al., 2011; Francisco et al., 2009). Taken together, the results from this study indicate that the engagement of PD-L1/PD-1 axis does deliver a suppressive, inhibitory signal to porcine lymphocytes and is involved in the induction of $CD4^{+}CD25^{HIGH}FoxP3^{+}$ Tregs in pigs.

Discussion

Dendritic cells are targets of infection by both PCV2 and PRRSV, though they are not considered main target cells for the replication of PCV2 (Chang et al., 2006). Previous studies have demonstrated that the presence of PCV2 in dendritic cells (DCs) does not alter the functionality of the myeloid DCs (Vincent et al., 2005), and the presence of virus in DCs has been suggested as a mechanism for viral transmission (Vincent et al., 2003). For these reasons, the effect of PCV2 and/or PRRSV infections on the phenotype of DCs is an important area of research. The results of this study provide insight into the maintained functionality of DCs during singular PCV2 infections, and perhaps some insight into the PCV2 and PRRSV co-infection necessity for the development of PCVAD. Increased PD-L1 expression has been shown to decrease T-cell responses and hinder viral clearance in other types of viral infections (McNally et al., 2013; Muhlbauer et al., 2006). Although we observed an increase in PD-L1 expression among MoDCs in response to singular PCV2 infection, CD86 was also significantly

increased. CD86 and PD-L1 are both members of the B7 family of proteins and the balance in the expression of these two proteins during viral infection can be crucial in the fate of the downstream adaptive immune response (Shen et al., 2010b).The observed increases in stimulatory marker CD86 expression among PCV2 infected MoDCs could help explain the efficient T-cell responses observed during singular PCV2 infection (Steiner et al., 2009) despite increases in PD-L1 expression. However, the increase in CD86 expression observed in singular PCV2 infected MoDCs is mitigated when PRRSV is introduced as a co-infecting pathogen.

Among the co-infected treatment groups, significant increases in PD-L1 expression were still observed at both the gene and protein levels; however, the expression of the stimulatory marker CD86 was significantly decreased. The PCV2b/VR-2385 co-infected MoDC treatment group also had the largest increase in IL-10 production. Because DCs not only activate T-cells but also negatively regulate T-cell responses (Banchereau and Steinman, 1998; Jonuleit et al., 2001; Mellman and Steinman, 2001; Selenko-Gebauer et al., 2003), this dendritic cell phenotype could deliver an overall inhibitory signal to the T-cells resulting in a dysfunctional adaptive immune response in cases of PCV2 and PRRSV co-infections.

One area of research that still requires clarification is the mechanism of lymphocyte depletion observed in cases of PCVAD (Darwich and Mateu, 2012). Significant increases in anergic and apoptotic cells were observed in the present study among lymphocyte populations that expressed the PD-1 gene at normal levels compared to PD-$1^{(-)}$ lymphocytes. Although lymphocyte depletion in cases of PCVAD is most likely the result of a combination of mechanisms including increased FasL activation, viral-induced cell lysis, and the destruction of the lymphoid architecture (Darwich and Mateu, 2012), the data from this study implicates the

observed increase in MoDC PD-L1 expression and the PD-L1/PD-1 axis as a potential additional mechanism for lymphocyte depletion in cases of PCVAD.

Previous research has shown that PCV2/PRRSV co-infected MoDCs induce regulatory T-cells (Cecere et al., 2012). This current study attempted to identify a potential mechanism for the increase in regulatory T-cells previously observed. Lymphocyte populations expressing normal levels of PD-1 had significantly higher percentages of $CD4^{+}CD25^{HIGH}FoxP3^{+}$ Tregs when compared to $PD\text{-}1^{(-)}$ lymphocytes. These findings correspond to studies in other animal species that demonstrate a role for the PD-L1/PD-1 axis in the induction and maintenance of Tregs (Amarnath et al., 2011; Francisco et al., 2009).

Taken together, the results of this study demonstrate the development of regulatory DCs during PCV2/PRRSV co-infection and describe a role for the PD-L1/PD-1 axis in swine lymphocyte anergy, apoptosis, and the induction of porcine $CD4^{+}CD25^{High}FoxP3^{+}$ Tregs. Although the results of this study require *in vivo* corroboration to confirm a potential role in the development of PCVAD, they do support the hypothesis that initial immune suppression may play a role in the pathogenesis of PCVAD.

Acknowledgements

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Tables

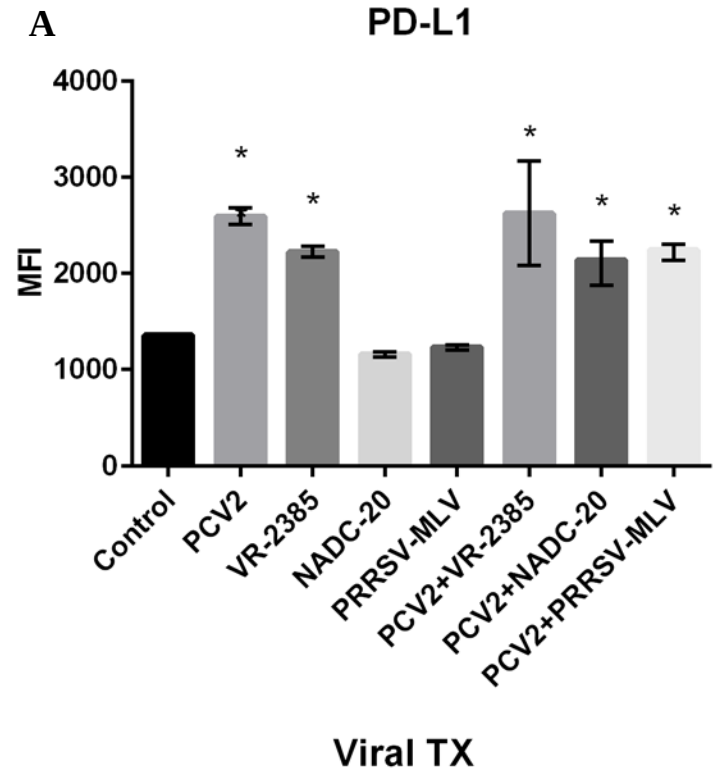
Table 5.1 Viral treatment groups for the analysis of PD-L1, CD86, and IL-10 MoDC expression

Group Number	PCV2b (MOI 1)	PRRSV (MOI 1)
1 (Control)	**Negative**	**Negative**
2	**NC-16845**	**Negative**
3	**NC-16845**	**VR-2385**
4	**NC-16845**	**NADC-20**
5	**NC-16845**	**MLV**
6	**Negative**	**VR-2385**
7	**Negative**	**NADC-20**
8	**Negative**	**MLV**

Table 5.2 Porcine PD-1 siRNA and negative control siRNA sequences

Porcine PD-1 siRNA Sequence	Sense Sequence CAGAGGACGGAGGAUGGACACUGCU Antisense Sequence AGCAGUGUCCAUCCUCCGUCCUCUG
Negative Control siRNA Sequence	Sense Sequence CAGGCAGGGAGGGUACACAUGAGCU Antisense Sequence AGCUCAUGUGUACCCUCCCUGCCUG

Figures


A
PD-L1
4000
3000
2000
1000
0
MFI
*
*
*
*
*
Control
PCV2
VR-2385
NADC-20
PRRSV-MLV
PCV2+VR-2385
PCV2+NADC-20
PCV2+PRRSV-MLV
Viral TX

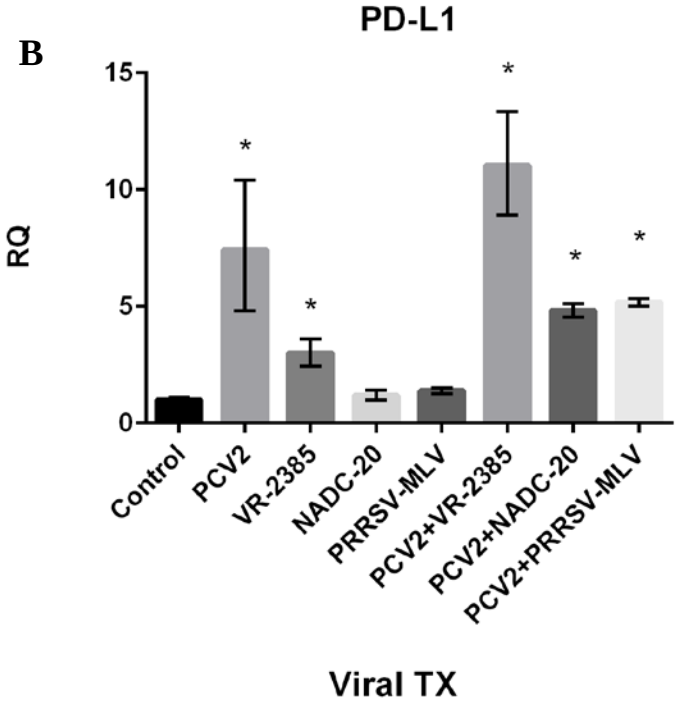
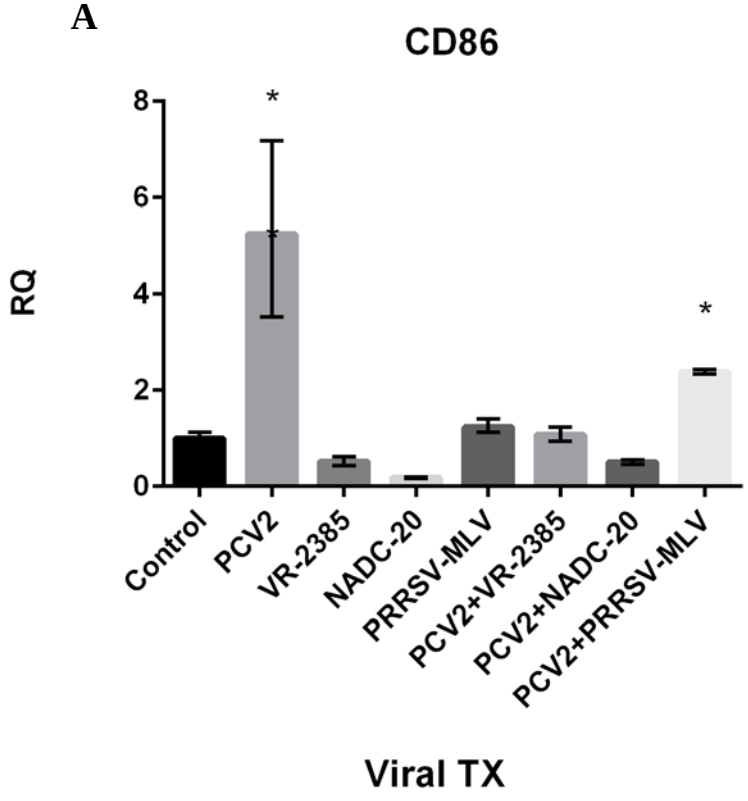

B
PD-L1
15
10
5
0
RQ
*
*
*
*
*
Control
PCV2
VR-2385
NADC-20
PRRSV-MLV
PCV2+VR-2385
PCV2+NADC-20
PCV2+PRRSV-MLV
Viral TX

Figure 5.1 A) Relative mRNA transcript levels of PD-L1 in porcine MoDCs after a 24 hour exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). **B)** Porcine MoDC PD-L1 surface expression levels determined by flow cytometry MFI after a 24 hour exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of 3 samples. Error bars indicate standard error.

A


CD86
RQ
8
6
4
2
0
*
*
Control
PCV2
VR-2385
NADC-20
PRRSV-MLV
PCV2+VR-2385
PCV2+NADC-20
PCV2+PRRSV-MLV
Viral TX

B

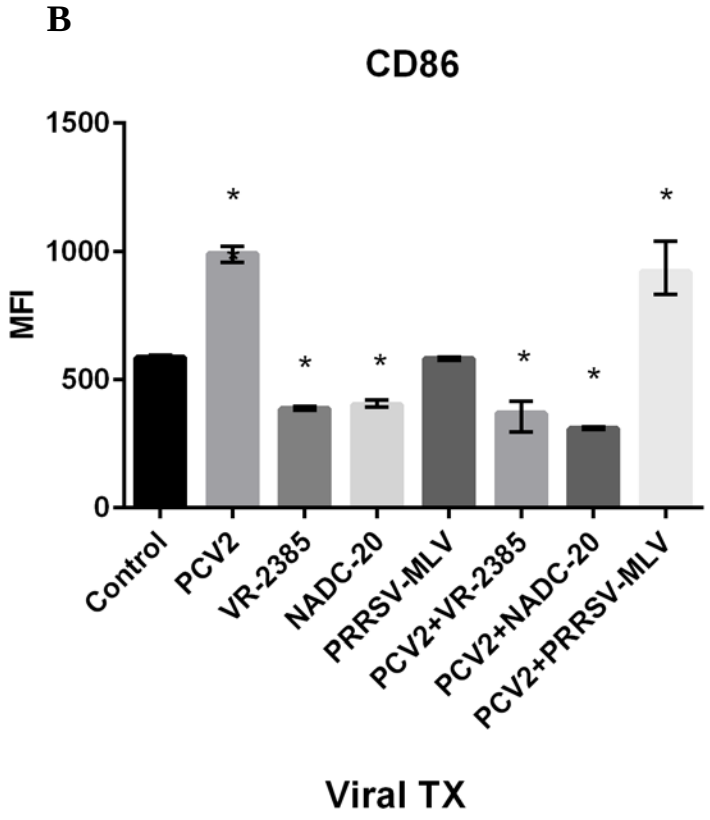

CD86
MFI
1500
1000
500
0
*
*
*
*
*
*
Control
PCV2
VR-2385
NADC-20
PRRSV-MLV
PCV2+VR-2385
PCV2+NADC-20
PCV2+PRRSV-MLV
Viral TX

Figure 5.2 A) Relative mRNA transcript levels of CD86 in porcine MoDCs after a 24 hour exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). **B)** Porcine MoDC CD86 surface expression levels determined by flow cytometry MFI after a 24 hour exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of 3 samples. Error bars indicate standard error.

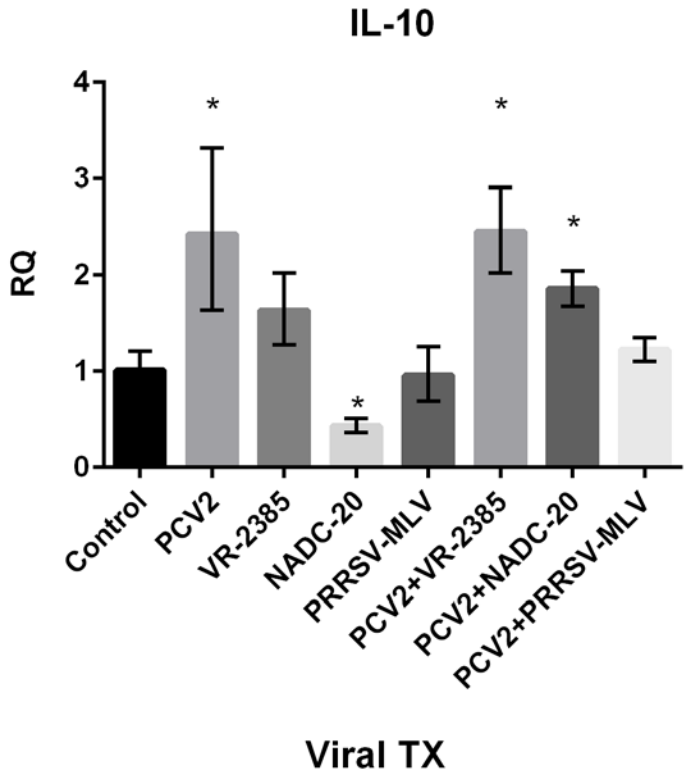


Figure 5.3 Relative mRNA transcript levels of IL-10 in porcine MoDCs after 24 exposure to the corresponding viral treatment group. * denotes statistical significance versus the control ($p<0.05$). Each bar represents the mean of 3 samples. Error bars indicate standard error.

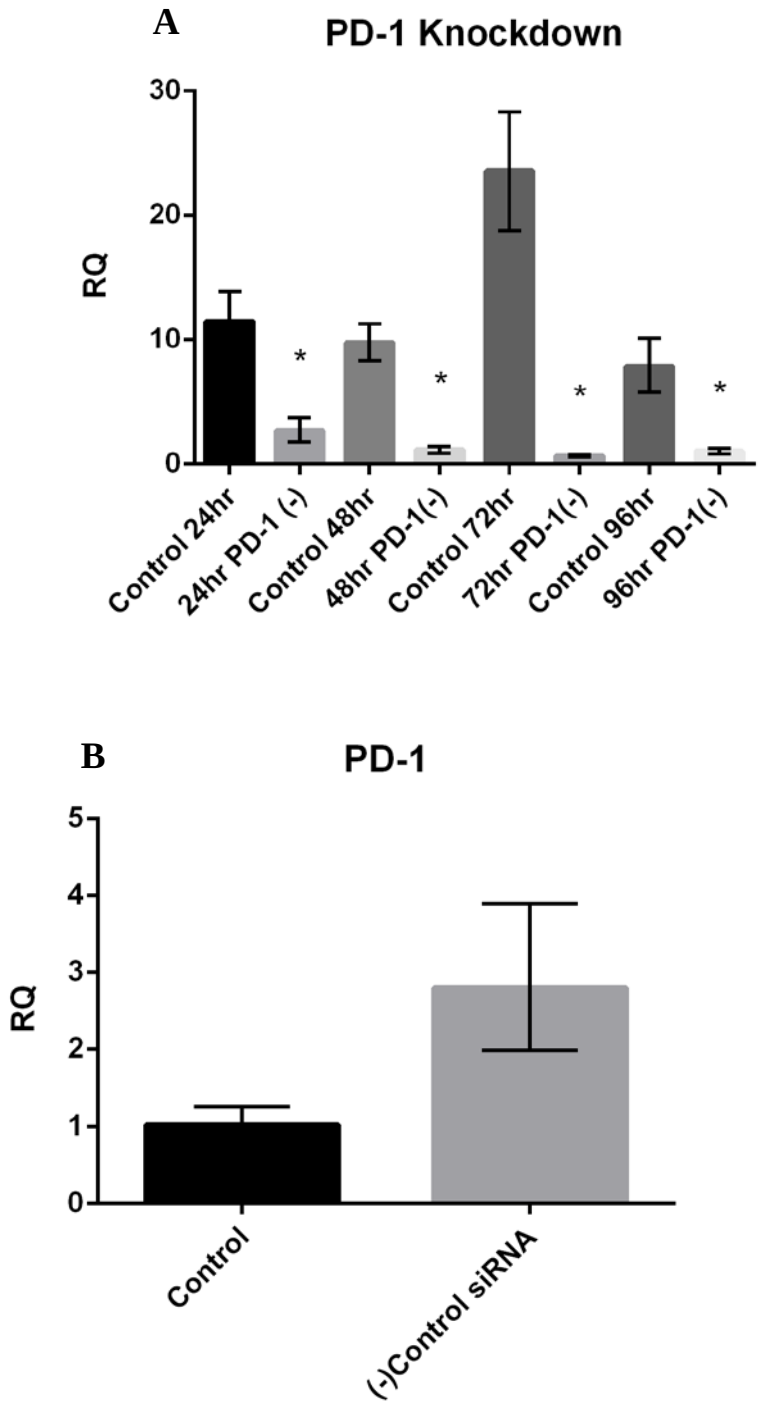


Figure 5.4 A) Expression levels of the PD-1 gene in mock treatment control porcine lymphocytes vs. PD-1 siRNA treated porcine lymphocytes as determined by qRT-PCR at 24hr,

48hr, 72hr, and 96hr post electroporation. * denotes statistical significance ($p<0.05$) versus the controls. Each bar represents the mean of three samples. Error bars indicate standard error. **B)** Expression levels of the PD-1 gene determined by qRT-PCR in in mock treatment control porcine lymphocytes vs. negative control siRNA treated porcine lymphocytes. Each bar represents the mean of 3 samples. Error bars indicate standard error.

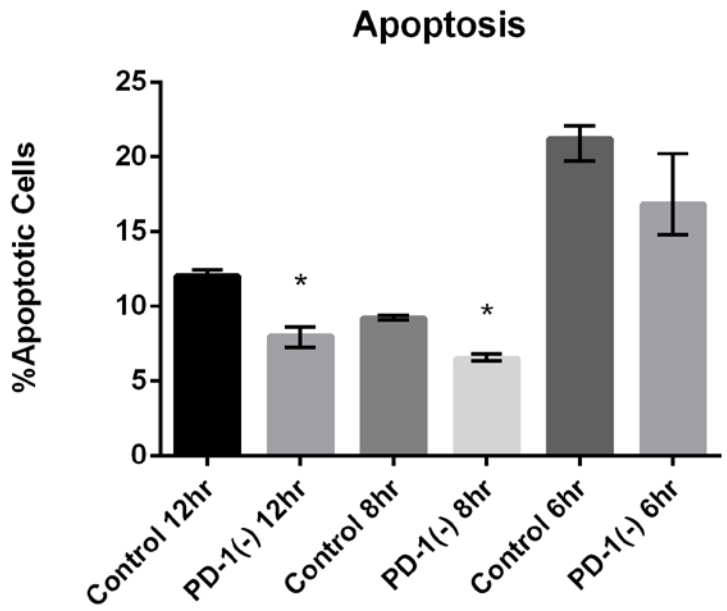


Figure 5.5 Percentages of apoptotic cells in PD-1defecient porcine lymphocytes vs. normal PD-1 expressing control porcine lymphocytes at 12hr, 8hr, and 6hr post MoDC exposure. * denotes statistical significance ($p<0.05$) versus the controls. Each bar represents the mean of 3 samples. Error bars indicate standard error.

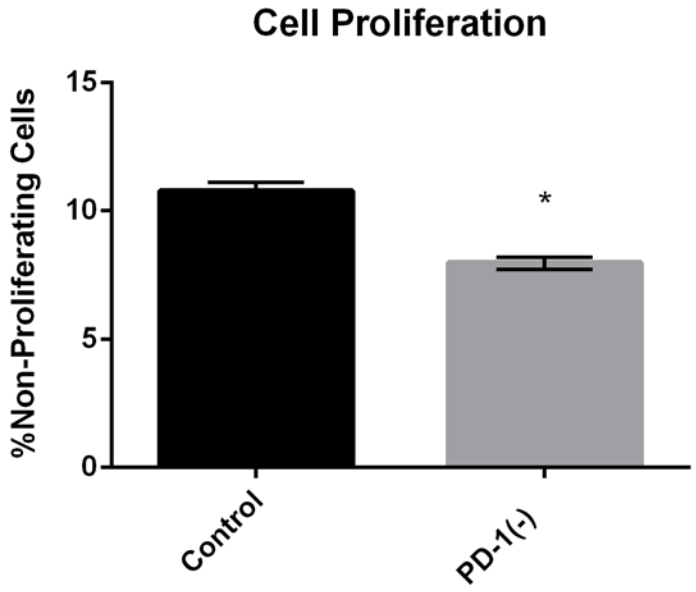


Figure 5.6 Percentages of non-proliferating cells in PD-1 deficient porcine lymphocytes vs. normal PD-1 expressing control porcine lymphocytes at 24hr post MoDC, 10ng/ml PHA-L exposure. * denotes statistical significance ($p<0.05$) versus the control. Each bar represents the mean of 3 samples. Error bars indicate standard error.

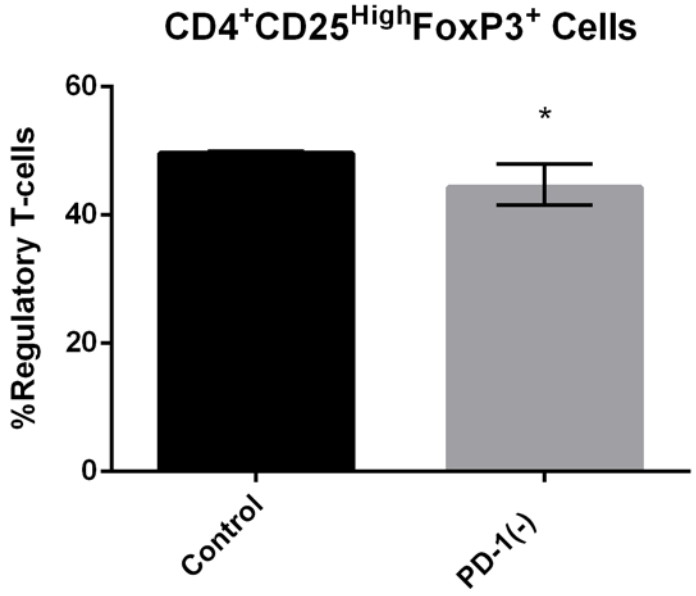


Figure 5.7 Percentages of $CD4^{+}CD25^{HIGH}FoxP3^{+}$ Treg cells in PD-1 deficient porcine lymphocytes vs. normal PD-1 expressing control porcine lymphocytes at 72hr post MoDC, 1ug anti-CD3, and 2ng/ml TGF-β exposure. * denotes statistical significance ($p<0.05$) versus the control. Each bar represents the mean of 3 samples. Error bars indicate standard error

Chapter 6: Summary and Conclusions

Modulation of the host immune system is thought to be crucial in the pathogenesis of PCVAD. Several immune suppression mechanisms have been implicated in the development of PCVAD including the induction of regulatory T-cells (Treg) which is suspected of contributing to viral persistence and disease severity (Cecere et al., 2012). The induction of Tregs by singular, strain-dependent PRRSV infection has been established *in vivo* (LeRoith et al., 2011; Silva-Campa et al., 2012), and the induction of Tregs was previously shown among T-cell populations co-cultured with PCV2 and PRRSV co-infected porcine monocyte derived dendritic cells (MoDCs) *in vitro* (Cecere et al., 2012).The objective in the first aim of this dissertation was to determine if Treg percentages increased *in vivo* and determine cytokine responses. Our hypothesis for the first aim of this dissertation was that the addition of PCV2 to a multiple viral challenge would result in an increase in the percentage of Tregs, increased IL-10 and TGF-β production, and decreased cell proliferation and IFN-γ production. The results showed that there was a significant increase in both the $CD4^{+}CD25^{High}FoxP3^{+}$ and $CD4^{+}CD8^{+}CD25^{High}FoxP3^{+}$ phenotypes of Tregs 14 days post viral challenge among unvaccinated pigs that were challenged with PCV2 in addition to other viruses. Although expression of IL-10 was increased in the lung tissue of pigs in this treatment group, the hypothesized increase in circulating TGF-β and IL-10 levels expected to accompany higher percentages of Tregs was not observed, nor was there a decrease in cell proliferation. Increased levels of circulating IFN-γ were also observed, contrary to the hypothesis, on days 14 and 21 post viral challenge. These findings suggest that a multi-faceted immune response may occur during PCVAD development that includes both immune suppression and immune stimulation, and that the timing of sampling is critical to establish the mechanism of immune modulation. Immune stimulation has previously been shown to increase

the effectiveness with which PCVAD can be reproduced (Allan et al., 2004; Nauwynck et al., 2012; Sanchez et al., 2004). This is most likely due the simple structure of PCV2 and its complete dependence on cellular polymerases for replication. The revised hypothesis based on the findings of this study is that there is an initial state of immune suppression that begins shortly after viral infection followed subsequently by a pro-inflammatory immune environment that favors rapid PCV2 replication. Because of the time sensitive nature of monitoring the proposed immune modulations, further experimentation with more frequent data collection is needed to confirm the revised hypothesis.

Programmed death ligand-1 (PD-L1) and its receptor programmed death-1 (PD-1) are important immunological research and therapeutic targets because of their ability to negatively affect lymphocyte activation. Over engagement of the PD-L1/PD-1 axis has been shown in human and murine species to cause lymphocyte apoptosis and anergy, and the induction and maintenance of Tregs (Amarnath et al., 2011; Francisco et al., 2009; McNally et al., 2013; Muhlbauer et al., 2006). Due to the experimentally established importance of the PD-L1/PD-1 axis in other viral infections (Barber et al., 2006; Day et al., 2006; Urbani et al., 2006), the second and third aims of this dissertation sought to investigate and describe altered PD-L1, IL-10, SLA-1, SLA-2, and CD86 expression among PCV2 and/or PRRSV infected MoDCs and examine whether the lymphocyte dysfunction associated with the PD-L1/PD-1 axis observed in other animal species occurs in pigs. Because of the association of both PCV2 and PRRSV with cells of the monocytic lineage, including dendritic cells (DCs), the resulting DC phenotype following PCV2 and/or PRRSV exposure is an important aspect of disease pathogenesis. We hypothesized that MoDC infection by PCV2 and/or PRRSV would increase PD-L1 and IL-10 expression while also decreasing co-stimulatory marker expression resulting in a regulatory

phenotype of MoDC. We further hypothesized that the engagement of the PD-L1/PD-1 axis in porcine lymphocyte and MoDC cell populations would result in an increase in lymphocyte anergy, apoptosis, and the induction of Tregs that has previously been shown in other animal species.

Our results provide the initial description of altered PD-L1 expression in porcine MoDCs following PCV2 and/or PRSV infection. Singular PCV2 infection and PCV2/PRRSV co-infection significantly increased MoDC PD-L1 expression at both the gene and protein levels, while singular PRRSV infection significantly increased MoDC PD-L1 expression at the gene and protein levels in a strain-dependent manner. These findings suggest that increased PD-L1 expression among antigen presenting cells (APC) following PCV2/PRRSV co-infection is a potential mechanism in the pathogenesis of PCVAD.

To further investigate potential APC dysfunction, we evaluated the expression levels of IL-10, swine leukocyte antigen-1 (SLA-1), swine leukocyte antigen-2 (SLA-2), and CD86 in MoDCs following PCV2 and/or PRRSV infections. The data indicates infection of MoDCs by virulent strains of PRRSV alone significantly decreased IL-10 expression in a strain-dependent manner and decreased the expression of SLA-1, SLA-2, and CD86. These results combined with the strain-dependent modulations of MoDC-PD-L1 expression add to the previous literature describing erratic and strain-dependent cell mediated immune response to PRRSV infections (Charerntantanakul et al., 2006; Lopez Fuertes et al., 1999; Meier et al., 2003). Singular PCV2 infection induced significant increases in IL-10 gene expression, while also significantly increasing gene and protein level expression of SLA-1, SLA-2, and CD86. The significant increases in co-stimulatory marker expression observed in these experiments is one possible mechanism explaining the effective activation of $CD4^+$ and $CD8^+$ T-cell responses previously

reported in cases of singular PCV2 infection that aide in viral clearance (Fort et al., 2009a; Steiner et al., 2009). Co-infection of MoDCs by PCV2 and virulent strains of PRRSV resulted in increased IL-10 gene expression and decreased SLA-1, SLA-2, and CD86 gene and protein level expression. Regulatory DCs are a heterogeneous cell population characterized not only by increases in IL-10 and regulatory molecule expression, but also decreases in co-stimulatory molecule expression (Gordon et al., 2014; Wolfle et al., 2011). Because the increases in MoDC IL-10 and PD-L1 expression were only accompanied with decreases in SLA-1, SLA-2, and CD86 in co-infected MoDCs, we hypothesize that co-infection of APCs by both PCV2 and a co-pathogen are required for the development of regulatory DCs, and this may be a mechanism in the underlying co-infection necessity of PCVAD. Although PCV2/PRRSV induced modulations to APC PD-L1, IL-10, SLA-1, SLA-2, and CD86 expression need to be confirmed *in vivo*, we hypothesize that the regulatory phenotype of MoDC observed in these studies following PCV2/PRRSV co-infection may translate to a mechanism of lymphocyte depletion and the overall pathogenesis in cases of PCVAD.

Several studies have demonstrated the impact of the PD-L1/PD-1 axis on lymphocyte responses in human and murine cells. To verify these findings in pigs, the final experiments of this dissertation evaluated anergy, apoptosis, and induction of Tregs in porcine lymphocytes with deficient PD-1 expression (PD-$1^{(-)}$) compared to porcine lymphocytes with normal levels of PD-1 expression. The results showed that normal PD-1 expressing porcine lymphocytes had significantly higher percentages of anergic and apoptotic cells, and a significant increase in the percentage of $CD4^{+}CD25^{High}FoxP3^{+}$ regulatory T-cells compared to PD-$1^{(-)}$ porcine lymphocytes. These findings supported our hypothesis that increased APC PD-L1 expression is involved in lymphocyte depletion and immune dysfunction in cases of PCVAD.

Although effective vaccination against PCV2 has decreased the negative health and economic impacts of PCVAD in commercial swine herds, the threat of new emerging genotypes and the ubiquitous nature of PCV2 around the world make elucidating the pathogenesis of PCVAD an important research topic. Over the past almost 20 years of PCV2 focused research many discoveries have led to a better understanding of PCV2 infections and PCVAD development. However, many aspects of disease progression at the cellular and molecular level remain elusive. Many of the findings of this dissertation require *in vivo* confirmation; however we believe this work adds significantly to the base of knowledge about PCV2 and PCVAD and will improve future therapeutic efforts.

Figures

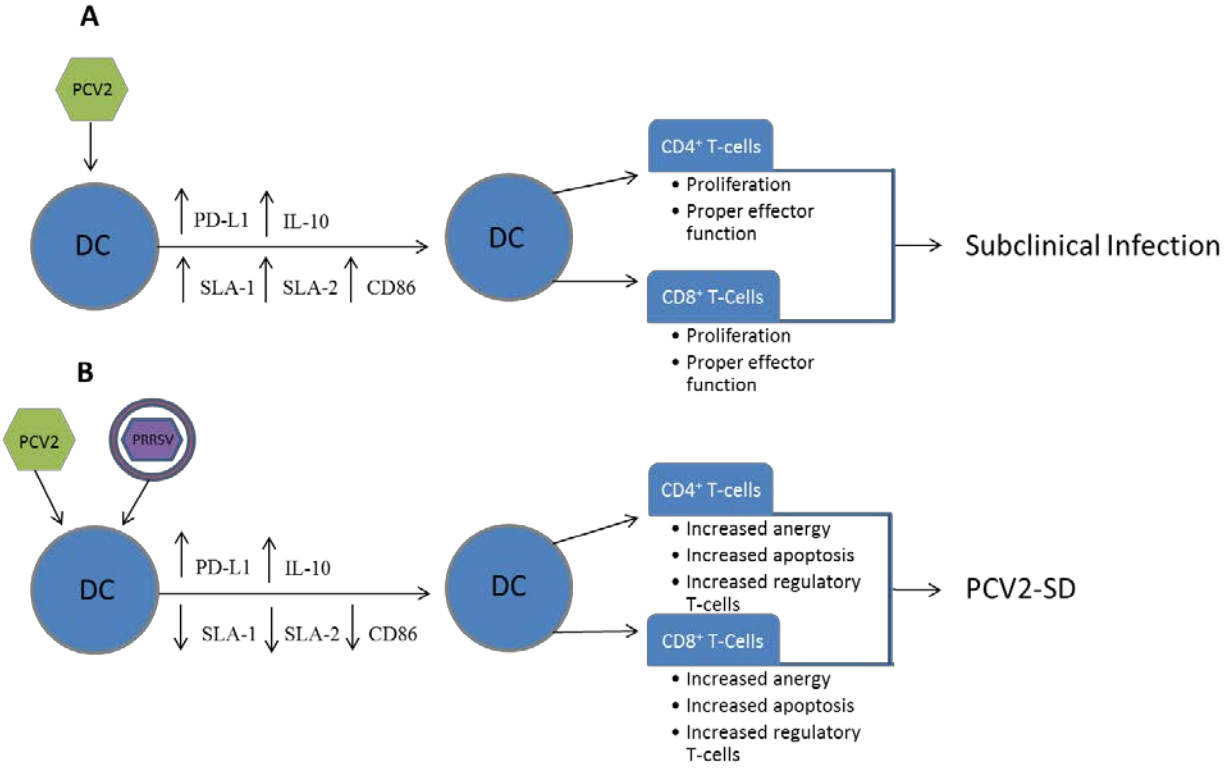
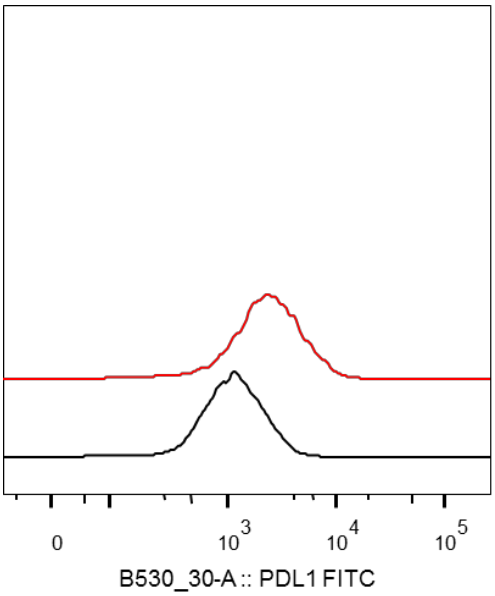


Fig. 6.1 Dendritic cell phenotypic changes and proposed T-cell responses following singular PCV2 infection of dendritic cells (A) and PCV2/PRRSV co-infection of dendritic cells (B).

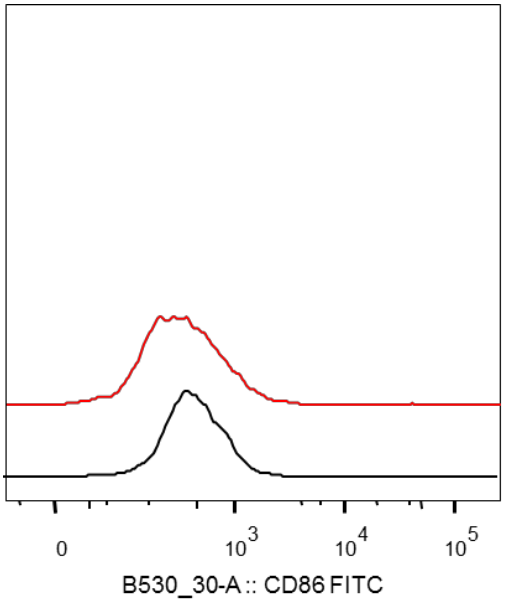
Appendices

Appendix A: Representative median fluorescent shift as determined by flow cytometry for MoDC PD-L1 expression in the control and PCV2+VR-2385 treatment groups.



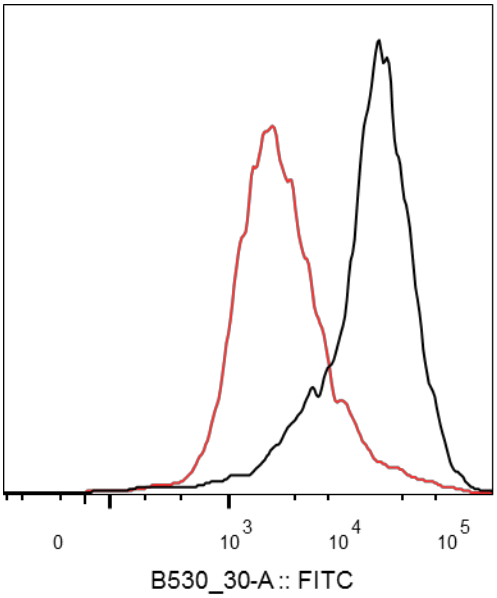
Sample Name	Subset Name		Median : B530_30-A
PD-L1_PCV2+VR-2385	Singlets	9315	2643
PD-L1_Control	Singlets	9411	1338

Appendix B: Representative median fluorescent shift as determined by flow cytometry for MoDC CD86 expression in the control and PCV2+VR-2385 treatment groups.



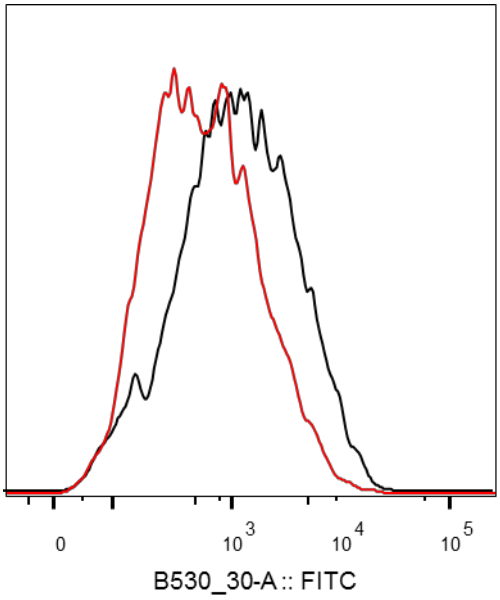
Sample Name	Subset Name	Count	Median : B530_30-A
CD86_PCV2+VR-2385	Singlets	5566	444
CD86_Control	Singlets	9480	585

Appendix C: Representative median fluorescent shift as determined by flow cytometry for MoDC SLA-1 expression in the control and PCV2+VR-2385 treatment groups.



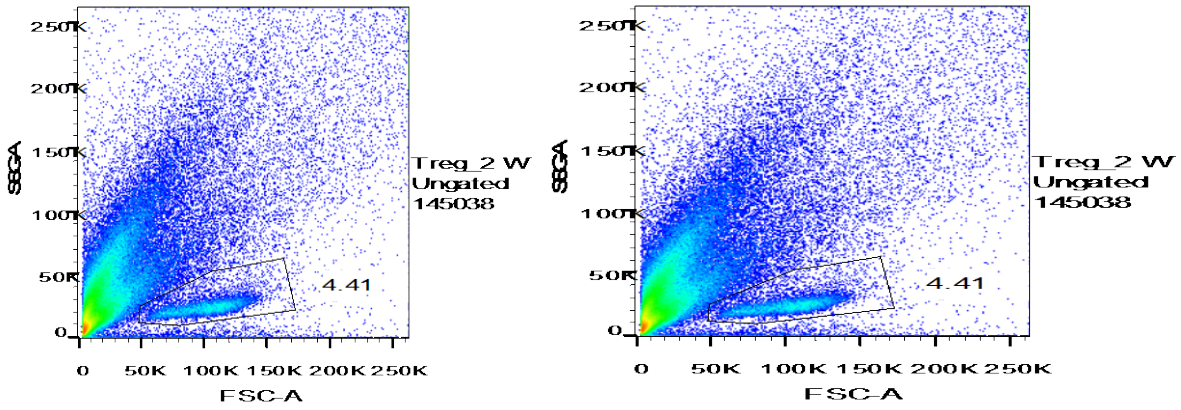
Sample	Name	Subset Name	Count	Median : B530_30-A
SLA-1	PCV2+VR-2385	Singlets	10778	3263
SLA-1	Control	Singlets	10310	19242

Appendix D: Representative median fluorescent shift as determined by flow cytometry for MoDC SLA-2 expression in the control and PCV2+VR-2385 treatment groups.

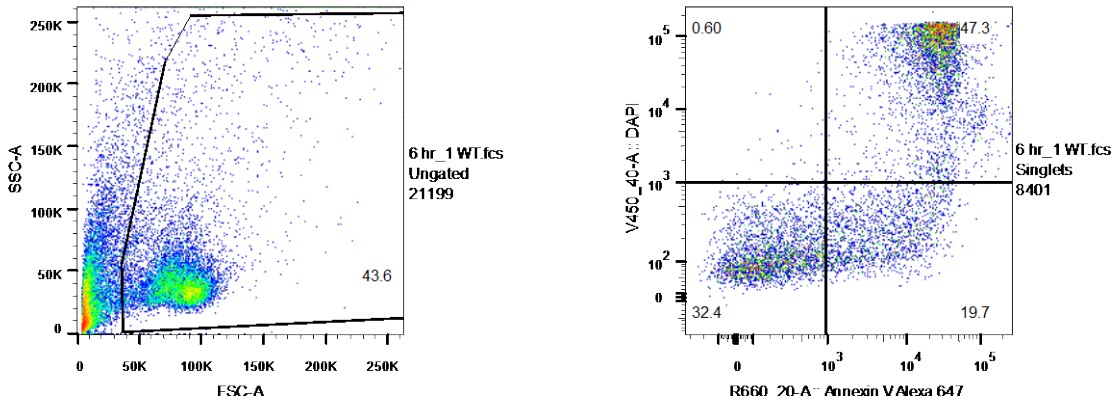


Sample Name	Subset Name	Count	Median : B530_30-A
SLA-2 PCV2+VR-2385	Singlets	10127	752
SLA-2 Control	Singlets	10027	1448

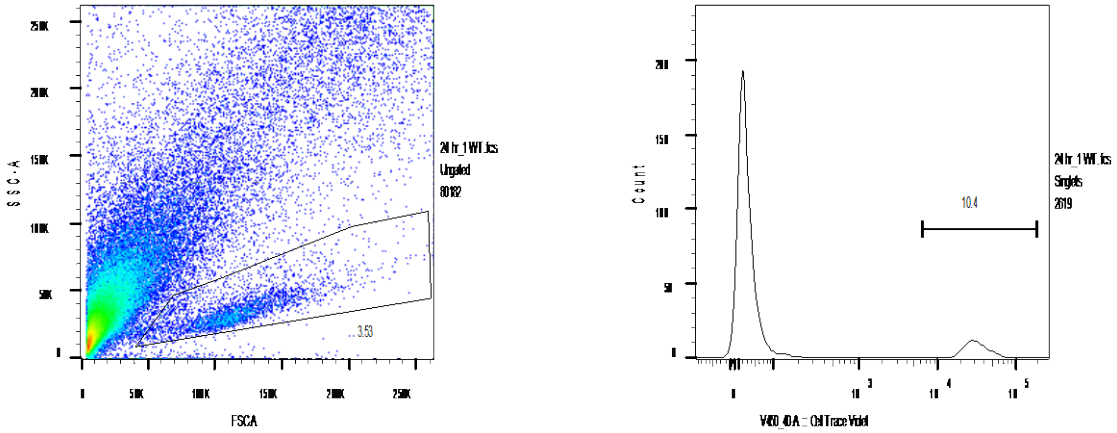
Appendix E: Representative flow cytometry profile of regulatory T-cell percentages following CD4 and CD25 gating. Cells are from a normal PD-1 expressing lymphocyte population 72hr post-MoDC exposure. Complete Media for the incubation was supplemented with 1 μg rat anti-porcine CD3 monoclonal antibody and 2ng /ml porcine TGF-β.



Appendix F: Representative flow cytometry profile of apoptotic cell percentage as determined by annexin-V and DAPI staining. Cells are from normal PD-1 expressing lymphocytes at 6hr post-MoDC exposure.



Appendix G: Representative anergic cell percentages as determined by flow cytometry. Cells are from a lymphocyte population with normal levels of PD-1 expression. Cell proliferation was determined 24hr post-MoDC exposure. Complete media for the incubation was supplemented with 10 µg/ml PHA-L. Cell proliferation was determined using Cell Trace™ violet cell proliferation kit (Life Technologies).



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